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Immunity to Methylcholanthrene-Induced Sarcomas[1]

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The history of attempts to immunize against cancer is one of long frustration. As a result of apparent failure during the past half century, it is the current consensus that immune mechanisms probably will be of little use in the control of this disease. This pessimistic outlook stems from the demonstration that immunity to common transplanted tumors of laboratory animals is not against the tumor *per se* but rather against antigens common to the tumor and the other tissues of the animal in which the tumor originated. Until the present time, immunity to transplanted tumors has proved to be identical with the immunity that is the unwanted but usual result of homografting or heterografting normal tissues (*1*). Immunity, unless directed specifically against antigens peculiar to cancer cells (or peculiar to etiologic agents), is useless in the prevention and therapy of cancer.

Despite these discouragements throughout a half century of endeavor, various investigators have persisted in seeking ways to immunize specifically against tumor tissues, and several have claimed success. Their experiments have been well reviewed (*1*, *2*). By various methods these investigators immunized mice of inbred strains against isologous tumors, *i.e.*, against tumors that had originated within the *same inbred strain.* (The prefix *iso-* is used to designate animals or tissues of supposedly identical genetic constitution, as produced by inbreeding.) Because members of an inbred strain were assumed to be genetically and thus antigenically identical, it was concluded that immunity must necessarily be directed against the tumor tissue *per se.* That these experiments did not command more attention is not surprising, since little or no evidence was advanced that the animals used were actually as homogeneous as assumed. Any inbred line may contain some residual heterogeneity, perhaps sufficient to account for the isologous immunity occasionally produced. Furthermore, the tumors usually had been transplanted

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many times, thus allowing ample opportunity for genetic changes in the mouse strain, the tumor, or both.

Our own interest in the problem was restimulated by the work of Foley (*3*). He not only demonstrated isologous immunity to a number of sarcomas induced with 3-methylcholanthrene (MCA) but also showed that a similar phenomenon could *not* be demonstrated with spontaneous mammary carcinomas. The latter observation suggested, for the first time, that the immunity discovered might be based upon some factor other than heterogeneity among the animals. In Foley's experiments, heterogeneity among the mice was demonstrably insufficient to allow immunization against the mammary tumors. Could heterogeneity then be invoked as an explanation of the immunity against the MCA-induced sarcomas? Either MCA-induced sarcomas were unusually susceptible to such immunity or the results depended on antigens peculiar to the tumor tissues rather than on antigenic differences among the mice.

The experiments reported in the present paper were undertaken to confirm, if possible, the findings of Foley and, if successful, to acquire evidence that would indicate whether the active antigens were peculiar to the tumor tissues.

Methods and Results

The first experiments were designed to produce isologous immunity against MCA-induced sarcomas in a number of inbred mouse strains or their F_1 hybrids. The animals were usually 3 to 6 months of age; both sexes were used. They were obtained from 3 sources: the animal-production facilities at the National Institutes of Health, the animal colonies of Dr. W. E. Heston, or those of Dr. H. B. Andervont.

The tumors were induced by subcutaneous implantation of a few crystals of MCA or of pellets of paraffin or cholesterol (2 to 3 mm. in diameter) that contained 10 or 5 percent MCA, respectively. Histologically, all were fibrosarcomas of similar type. Fourteen such tumors were produced and used. When a tumor arose it was excised, and a small fragment was transplanted with a 12-gauge trocar to the subcutaneous tissues of the dorsum of mice of the isologous strain.

The growths resulting from this immunizing first transplant often did very poorly but were allowed, in those animals in which growth occurred, to attain a size of approximately 1 cm. At that time, each first-transplant-generation tumor was strangled by successively tighter ligatures, following closely the technique used by Foley (*3*). In later experiments of this series, simple surgical excision was substituted for ligation without apparently altering the results.[5] Two days to 2 weeks after final eradication of the first-transplant-generation tumors, each animal, including those in which the tumor had not grown, was tested for tumor immunity by subcutaneously implanting a second fragment of tumor by trocar. (The time interval before implantation of the second fragment of tumor, within the limits employed, was not a significant variable.) The second, or chal-

[5] No strangulation or surgical procedure was employed in animals in which the first transplant failed to grow.

lenge, implant was obtained from an animal bearing a first-transplant-generation growth from the same MCA-induced tumor that had been used for the previous, immunizing, implantation. The second implant was usually placed in the subcutaneous tissues of the abdomen to avoid confusion with possible recurrences of the immunizing growth. Suitable control animals, usually previously untreated (but in 1 experiment subjected to strangulation of a patch of dorsal skin), were likewise challenged. The challenge inoculations were always given alternately to mice of the control and experimental groups.

The growth of tumors from the challenge implants was carefully recorded. It was noted that, even in the control groups, the tumors usually did not grow in all mice, and regression after temporary growth was not uncommon. (This difficulty was not encountered with tumors of subsequent transplant generations.)

In the results given in table 1, only tumors that grew progressively (*i.e.*, without regression) are recorded. The table shows that isologous tumor immunity was produced by contact with tumor tissue obtained from 11 of 14 MCA-induced fibrosarcomas. Isologous immunity to 1 of the 3 tumors that gave negative results was produced in a subsequent transplant generation by immunizing the recipients twice.

Evidence indicating that the isologous immunity was not dependent upon heterogeneity among the mice was obtained in a second series of experiments, similar to the preceding, except that the tumors used were not induced by MCA but were spontaneous fibrosarcomas obtained from

TABLE 1.—*Results of attempts to produce isologous immunity to transplanted MCA-induced fibrosarcomas*

Tumor no.	Strain	Sex	Method of removal of immunizing tumor	Growth of challenge tumors					
				Immunized mice			Controls		
				No. tumors	No. mice	Percent	No. tumors	No. mice	Percent
H	BALB/cAn	♂	No growth	0	39	00	6	38	16
G	BALB/cAn	♂	Excision	5	27	19	33	37	89
A	C3Hf/He	♀	Excision	3	18	17	18	21	86
38	C3H	♀	Excision	3	11	27	11	11	100
K	DBA/2	♂	Excision	2	8	25	5	6	88
E	C3Hf/He	♂	Ligation	2	13	15	14	15	93
C	C3Hf/He	♀	Ligation	8	14	57	18	19	95
177	BALB/cAn	♂	Ligation	3	32	9	15	32	47
170	(C × DBA)F_1	♂	Ligation	9	14	64	10	10	100
J	C3H/He	♀	Excision	3	11	27	11	11	100
L	DBA/2	♂	Excision	0	7	00	6	8	75
SUBTOTAL				38	194	20	147	208	71
9	DBA/2	♂	Excision	7	7	100	7	7	100
8	DBA/2	♂	Excision	7	7	100	8	8	100
61	C3Hf/He	♀	Excision	8	8	100	7	8	87
SUBTOTAL				22	22	100	22	23	96
GRAND TOTAL				60	216	30	169	231	73

Dr. W. E. Heston and Dr. H. B. Andervont, of the National Cancer Institute. Such sarcomas arise occasionally in mice of the C3Hf/He and C3Hf/An lines. Over a period of 2 years, it was our good fortune to obtain 7 of these tumors for testing. In each case, the experiment was initiated with previously untransplanted tumor, and the resulting growths were excised.

After primary transplantation, the spontaneous tumors grew in a larger percentage of animals than the MCA-induced tumors; but their rates of growth were quite similar. The spontaneous fibrosarcomas were histologically indistinguishable from their MCA-induced counterparts.

The results of the challenge by implants derived from these spontaneous tumors are recorded in table 2. The difference in the results obtained from those with the MCA-induced tumors is striking. With the tissues from the spontaneous tumors no detectable isologous immunity was produced.

TABLE 2.—*Results of attempts to produce isologous immunity to transplanted fibrosarcomas of spontaneous origin*

Tumor no.	Strain	Sex	Growth of challenge tumors					
			Immunized mice			Controls		
			No. tumors	No. mice	Percent	No. tumors	No. mice	Percent
109p	C3Hf/He	♀	13	13	100	9	9	100
109b	C3Hf/He	♀	2	14	14	2	5	40
109c	C3Hf/An	♀	9	12	75	7	8	88
109d	C3Hf/An	♀	11	12	92	7	7	100
109e	C3Hf/He	♀	6	6	100	6	6	100
109f	C3Hf/He	♀	25	26	96	26	26	100
187p	C3Hf/He	♀	28	30	93	25	29	86
TOTAL			94	113	83	82	90	91

This series of experiments not only suggests a basic difference in antigenicity between MCA-induced and spontaneous fibrosarcomas but also suggests that the mice of at least 1 of the strains used (C3Hf/He) were highly homogeneous. Further substantiation of the latter point was obtained with mice of another of the inbred strains in a third series of experiments in which normal rather than tumor tissue was used.

Forty male mice of the BALB/cAn strain, 3 months of age, were paired, and 2 × 2 cm. skin grafts were exchanged between the members of each pair. [The full-thickness skin-grafting technique has been described in detail (*4*).] Twenty days later the skin grafts were excised and the denuded graft bed was again covered—partly by an autograft and partly by a second isograft from the same donor. The "second-set" grafts were then observed for more than 3 months. No difference could be detected between the survival of the "second-set" grafts and the autografts, all of which were successful.

The lack of detectable immunity to skin isografts in strain BALB/cAn mice and to tumors of spontaneous origin in C3Hf strains provides strong,

albeit indirect, evidence that the immunity against the tumors descended from MCA-induced sarcomas was not dependent upon heterogeneity among the mice but was indeed directed specifically against an antigen associated with the tumor. However, the fourth series of experiments provides evidence of a more direct type and also suggests that the effective antigen or antigens associated with the MCA-induced tumors were limited to the tumor tissues.

That the isoantigens of the MCA-induced sarcomas were peculiar to the tumor tissues was demonstrated directly by showing that, although implants of an MCA-induced tumor could immunize isologous mice against subsequent implants of the same tumor, normal tissues from the very same mouse in which the tumor had been originally induced could *not* immunize against the tumor. Conversely, it was also shown that implants of MCA-induced tumor tissue could not immunize isologous mice against skin grafts obtained from the very animal from which the tumor had been originally obtained. Apparently, tumor tissue, and only tumor tissue, could either elicit or respond to the isologous immunity. Thus, it is reasonable to conclude that the effective antigen or antigens were peculiar to and confined within the tumor tissues *per se* rather than shared with the other tissues of the animal of origin.

The normal tissues used for attempted immunization against the implants descended from the MCA-induced tumors were a finely chopped mash consisting of a mixture of spleen, liver, heart, lymph node, and skeletal muscle. The dose delivered subcutaneously to each experimental mouse filled a 12-gauge trocar for 1 cm. of its length. No attempt was made subsequently to strangulate or excise this tissue implant, since the previous work had shown this procedure to be superfluous when the immunizing implant failed to grow.

In the experiments in which the challenge was made with normal tissue, 1 cm.^{2} full-thickness skin grafts were employed. The grafts were limited to this size because, in each experiment, all of the grafts had to be obtained from 1 mouse, the animal in which the tumor had originally been induced. Because the fate of such small skin grafts is sometimes difficult to evaluate, a series of isografts of similar size was established in previously untreated control animals to aid the evaluation.

The data for the 2 types of experiments (immunization with normal tissues against implants of MCA-induced tumor tissue and immunization with MCA-induced tumor tissue against isologous skin grafts) are summarized in table 3.

Taken collectively, the various experiments that have been described up to this point indicate that most of the MCA-induced tumors contained an antigen, or antigens, peculiar to the tumor that was capable of eliciting an isologous immune reaction directed specifically against the tumor tissues.

Several of the MCA-induced sarcomas were tested for persistence of the isoantigenic quality in subsequent transplant generations. The results of these experiments are recorded in table 4. In general, antigenicity per-

TABLE 3.—*Results of tests for common isologous antigens between MCA-induced sarcomas and normal tissues**

Tumor no.	Transplant generation of tumor used for immunization and for challenge	Strain	Sex	Challenge tissue	Growth of challenge tissue implants								
					Mice immunized with tumor tissue			Mice immunized with normal tissue			Control mice (previously untreated)		
					No. of takes	No. of mice	Percent	No. of takes	No. of mice	Percent	No. of takes	No. of mice	Percent
H	3–4	BALB/cAn	♂	Tumor	1	36	3	34	40	85	30	40	75
H	1	BALB/cAn	♂	Skin graft	19	20	95	—	—	—	18	20	90
G	3–4	BALB/cAn	♂	Tumor	1	20	5	—	—	—	24	24	100
G	1	BALB/cAn	♂	Skin graft	11	12	92	—	—	—	20	20	100
J	2–3	C3H/He	♀	Tumor	3	11	27	11	11	100	11	11	100
TOTAL				Tumor	5	67	8	45	51	88	65	75	86
				Skin graft	30	32	94	—	—	†	38	40	95

*Normal tissues in every instance were obtained only from the mouse in which the tumor had originally been induced.
†The regular success of "second-set" isologous skin grafts was noted in discussing the previous experiment. *See* text.

sisted in the later transplant generations of the tumors tested, with 1 significant exception. One line of tumor "G" lost the antigenic property between the fifth and sixth transplant generations. It is possible that the other tumors might have exhibited a similar loss had they been observed through a larger number of generations.

The next question investigated was whether all the MCA-induced tumors contained identical antigens or whether each was antigenically distinct. The cross-reaction studies were done with tumors in the second to fourth transplant generations. The experiments were patterned after those of the first group, except that the immunizing and challenge implants, in each experiment, were descended from tumors of different

TABLE 4.—*Persistence of isologous immunity in later tumor transplant generations*

Tumor no.	Strain	Sex	Transplant generation of immunizing tumor	Transplant generation of challenge tumor	Growth of challenge tumors					
					Immunized mice			Controls		
					No. tumors	No. mice	Percent	No. tumors	No. mice	Percent
H	BALB/cAn	♂	1	2	0	39	00	6	38	16
			2	3	1	36	3	30	40	75
			3	4	0	44	00	32	44	73
A	C3Hf/He	♀	1	2	3	18	17	18	21	86
			4	5	1	21	5	18	19	95
G	BALB/cAn	♂	1	2	5	27	19	33	37	89
			3	4	1	20	5	24	24	100
			4	5	18	40	45	21	21	100
			4	5	19	29	66	25	31	81
			5	6	24	32	75	18	18	100
			6	7	15	16	94	22	22	100
K	DBA/2	♂	1	2	2	8	25	5	6	83
			3	4	2	27	6	24	24	100
L	DBA/2	♂	1	2	0	7	00	6	8	75
			3	4	0	32	00	24	24	100

origin. To demonstrate the continuing ability of the tumors to elicit or respond to autoinduced isologous immunity, control groups were run concurrently with the experiments in some cases. In others, the subsequent immunologic behavior of the tumors was utilized to control this factor. All the immunizing implants were excised.

The results of these experiments are shown in table 5. There is evidence of incomplete cross-reaction between certain tumors. The only conclusion one can reach at present is that the MCA-induced tumors tended to be immunologically distinctive but that there were partial cross-reactions between some of them. It is evident that more data will be required before the antigenic interrelationships of MCA-induced tumors can be assessed with confidence.

TABLE 5.—*Cross-immunity between MCA-induced tumors**

Tumors and mouse strains	Generation	Immunizing and challenging tumors of different origin				Immunizing and challenging tumors of identical origin				No prior immunization—challenge only			
		Immunizing tumor	Challenge tumor	No. takes	No. mice	Immunizing tumor	Challenge tumor	No. takes	No. mice	Immunizing tumor	Challenge tumor	No. takes	No. mice
L-DBA/2	3	L	K	9	24	K	K	2	24	—	K	24	24
K-DBA/2	3	K	L	2	24	L	L	0	24	—	L	24	24
G-BALB/c	3	H	G	34	40	G	G	1	20	—	G	33	37
H-BALB/c	2	G	H	2	11	H	H	0	39	—	H	6	38
H-BALB/c	4	G	H	12	43	—	—	—	—	—	H	32	47
G-BALB/c	4												
A-C3Hf/2	4	A	H	10	20	H	H	1	36	—	H	34	53
H-BALB/c	3	—	—	—	—	A	A	1	21	—	A	18	19
TOTAL				69	162			5	164			171	242

*All experiments performed in mice isologous with respect to the challenge tumor implants.

Discussion

The experiments presented in this paper, together with the findings of Foley (*3*), demonstrate that most MCA-induced tumors possessed an isologous antigenicity not shared by the spontaneous tumors tested nor by the normal tissues of the mouse.[6]

That MCA itself was not a component of the isoantigens is suggested by the persistence of antigenicity through a number of transplant generations.

The observations that 2 MCA-induced tumors were found that did *not* possess demonstrable isoantigens and that various tumors had different antigens suggest that the altered antigenicity was not an intrinsic part of the carcinogenic process but rather an incidental concomitant change. That the isoantigens were not produced as a part of the malignant process *per se* is also indicated by the fact that 1 isoantigenic MCA-induced tumor lost its antigenicity after numerous transplantations. Since there would have been considerable selective pressure favoring cells that lost the antigenic alteration but kept the purely malignant change, one may speculate that all the tumors, if transplantation had been continued for a sufficient length of time, might have exhibited similar behavior.

MCA is a potent biologic agent, being a mutagen as well as a carcinogen. It is, therefore, reasonable to hypothesize that, under the impact of this agent, a number of different heritable alterations might occur almost simultaneously in a single cell. Among these might be changes resulting in altered antigenicity, as well as other independent alterations resulting in malignancy.

If this hypothesis is correct, it would be reasonable to assume that the more violent and fast-acting a carcinogen, the more likely the occurrence of simultaneous antigenic and neoplastic changes. Slow-acting carcinogens (such as the mammary-tumor milk agent) could hardly be expected, on the basis of probability, to produce more than 1 cellular alteration at a time. It is also possible that viral carcinogens may be more specific in action than MCA.. For these reasons, "spontaneous" tumors might not exhibit isoantigenicity, except in rare instances.

Malmgren *et al.* (*6*) showed that all the carcinogens they tested (including MCA) produced a significant depression in antibody production. Similar findings have been reported by Davidsohn *et al.* (*7*). If cells in which a carcinogenic change has been produced are also antigenically altered, antibody depression by the carcinogen would give these cells a better chance to develop into tumors. In the absence of antibody depression by the carcinogen, relatively few isoantigenic tumors could be expected to develop. It is also of interest, in connection with the role of antibody depression, that administration of trypan blue has been shown to enhance carcinogenesis by MCA and dibenz[*a*,*h*]anthracene (*8*).

The fact that some cross-reactions were found among various tumors induced by MCA suggests that mice may be immunized to a significant degree against MCA carcinogenesis by using MCA-induced tumor

[6] Baldwin also has reported 3 tumors apparently possessing isoantigenicity (*5*).

tissue as antigen. Preliminary experiments in this laboratory, which will be reported in detail in a subsequent publication, indicate that this is actually the case. However, the apparent lack of complete cross-reaction among some tumors would seem to limit the possible practical application of these methods. Furthermore, it has not yet been determined whether other chemical carcinogens will also produce isoantigenic tumors and, if so, whether these would cross-react with MCA-induced malignancies.

Summary and Conclusions

Twelve of 14 subcutaneous fibrosarcomas induced with 3-methylcholanthrene (MCA) produced immunization within the inbred strain of mice in which each had originated. Isologous immunity was not produced by any of 7 histologically similar spontaneous sarcomas. Normal tissues from the same mouse in which the iso-immunizing MCA-induced tumor had originated did not elicit or respond to the isologous immunity. It is concluded that the antigens were peculiar to and specific for the tumor tissue. The antigenicity persisted through a number of tumor transplant generations, except in 1 instance where a tumor lost the antigenic property between the fifth and sixth transplant generations. Partial cross-reactions were observed among the tumors. These results suggest that immunization against some types of carcinogenesis, using tumor tissue as antigen, may be feasible.

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Development of Tumors in Fetal and Adult Lung Transplants [1]

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In a previous study (*1*) it was observed that lung from strain A mice, genetically susceptible to pulmonary tumors, and lung from strain C57L mice, genetically resistant to pulmonary tumors, retained their respective susceptibility and resistance to tumor formation when transplanted into the same host, the F_1 hybrid of these 2 strains. After the hosts had received an intravenous injection of dibenz[*a*,*h*]anthracene, many tumors appeared in the strain A lung transplants but few occurred in the C57L lung transplants. Similar results were reported by Shapiro and Kirschbaum (*2*) with the susceptible Bagg albino strain and the resistant strain DBA, using urethan as the carcinogen.

In an earlier analysis of the genetics of pulmonary tumors (*3*), it had been estimated that strain A differed from strain C57L by at least 4 gene pairs that caused strain A to be highly susceptible and strain C57L to be highly resistant. The fact that susceptibility and resistance were retained in the lungs when they were transplanted into a common host indicated that these genes controlled the degree of susceptibility through actions limited to the lungs rather than through some general systemic mechanism.

After these original observations, several questions remained unanswered. Was this irreversible fixation of the degree of susceptibility limited to postnatal lung, or was the degree of susceptibility established in the lung of the fetus so that it could not be reversed by transplantation into a genetically different host? Could results be obtained for spontaneous tumors similar to those observed when a carcinogen was injected into the host? What was the effect of transplantation *per se* upon the development of tumors in either the adult or fetal lung transplanted into mice of the same strain as that of the donor? In order to answer these and related questions, a study was outlined to observe occurrence of tumors in genetically susceptible and genetically resistant lung transplants, from fetuses and adults, in hosts injected with a carcinogen and in non-

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injected hosts. Smith (*4*) had demonstrated the neoplastic potentialities of transplanted fetal lung that had been minced and then mixed with methylcholanthrene before introduction into the host.

Materials and Methods

Mice of the susceptible strain A and of the resistant strain C57L were used as both donors and recipients of lung transplants. In addition, the recipients included their reciprocal F_1 hybrids, AL F_1 (with A mothers and C57L fathers), and LA F_1 (with C57L mothers and A fathers). Lung from both adult and fetal strain A mice was transplanted into other strain A mice and into both types of F_1 hybrids. Likewise, lung from both adult and fetal strain C57L mice was transplanted into other strain C57L mice and both types of F_1 hybrids. All recipients were approximately 30 days of age at the time of transplantation, and the adult lung transplants were taken from donors of comparable age. The fetal transplants were from 12- to 18-day fetuses. Each adult lung transplant was 1 of about 30 fragments of the lung, which had been cut with a pair of fine scissors, whereas a whole lobe of the fetal lungs was used for each transplant. The transplants were placed subcutaneously in the right and the left axillae of each host with a 12-gauge trocar. The hosts were approximately equally divided as to sex, and the donor of the adult lung transplant was always of the same sex as the recipient. No attempt was made to sex the fetal donors.

Each host-transplant combination included one group of hosts that, 30 days after receiving the transplant, were injected intravenously with 0.5 mg. dibenz[*a*,*h*]anthracene dispersed in 0.5 cc. water and one group of hosts that were not injected with the carcinogen, except for the AL F_1 hybrids with fetal A lung transplants and those with fetal L lung transplants. These AL F_1 hybrids did not include groups injected with the carcinogen.

Throughout the experiment the mice were kept segregated by sex in plastic cages, with 8 mice in each cage. They were given Derwood pelleted food and tap water *ad libitum*.

All mice were kept until moribund either because of tumors in their own lungs, in those given carcinogen, or because of conditions associated with advanced age in the others. At that time they were killed; the lung transplants were recovered; their own lungs were examined for tumors; and any tumors occurring at other sites were noted. Some of the animals were found dead in their cages, and, when possible, they were also examined for tumors and their transplants were recovered. The tumors and the lung transplants were fixed in Fekete's modification of Tellyesniczky's fluid (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part), embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histologic examination. The adult lung transplants, which did not increase in size, were serially sectioned, whereas the fetal transplants, which grew considerably, were sectioned semiserially.

Histology of Lung Transplants

The findings on histologic examination of the transplants and of the grossly obvious lesions in the host animals are presented in table 1.

The pulmonary tumors observed in the transplanted lungs were identical with those described repeatedly in the past (*5*). Their occurrence in the various host-transplant combinations is discussed in a subsequent section. Classification of host tumors of reticular tissue follows that proposed by Dunn (*6*), and the classification of mammary tumors follows that also proposed by Dunn (*7*).

Several other lesions were observed rather frequently in the transplanted lungs. The lesion designated as bronchial proliferation fulfills the criteria set forth in the paper by Heston and Dunn (*1*). In some serial sections continuity could be traced between the cylindrical cells lining alveolar spaces and identical-appearing cylindrical cells lining the smaller bronchioles (fig. 2). This may be interpreted as a suggestion that these cells represent a downgrowth of bronchiolar epithelium. The mere occurrence of continuity, however, does not prove this contention. The occurrence of bronchial proliferations in the various groups of transplants is indicated in table 1. As in the former study (*1*), the incidence was greater in transplanted strain C57L lung than in transplanted strain A lung. It is also noteworthy that the incidence was greater in the transplanted adult lung than in the transplanted fetal lung. About 75 percent of the transplants of adult lung revealed bronchial proliferation, whereas only about 35 percent of the fetal lung transplants showed this lesion. The interpretation of this finding is not immediately obvious; perhaps bronchial proliferation was a response to a stimulus (chemical or microbiologic) present in the atmosphere. This agent might have already been present in the adult lung fragments at the time of transplantation. If such an agent exists, it might reach the fetal lung transplants by absorption into the circulatory system of the host animals through the respiratory route. This hypothesis assumes that the lesion is a response to a nonspecific irritative process, which seems to be the most likely stimulus to this proliferation. On histologic grounds there is no doubt that the process is benign and of the order of hyperplasia rather than neoplasia.

Lymphocytic infiltration in these transplants was extremely common. It was present equally in fetal and adult lung transplants, and the incidence was not appreciably influenced by the strain or sex of the host animal or by treatment or lack of treatment with carcinogen. Much of this probably represents simply the appearance of lymphoid nodules that were present in the lung transplants at the time of transplantation. It is possible that the lymphoid infiltration of the more massive type represents a host response to the transplanted tissue.

Not infrequently epidermoid cysts were also observed in these transplants. They were seen in only 2 transplants of adult lung, whereas they were present in 30 transplants of fetal lung. In occasional transplants, the epidermoid cysts could be traced in continuity with fragments of transplanted esophagus. However, the majority of the cysts occurred in

TABLE 1.—*Occurrence of tumors and other lesions in the various lung transplants and hosts*

Donor of transplant	Strain of host	Sex of host	Injected	No. of hosts	No. with tumors in own lungs	Avg. age of hosts (mos.)	No. hosts with given no. tumors in lung transplants									No. hosts with given lesion in lung transplant					Other tumors and lesions
							0	1	2	3	4	5	6	7	9	BP*	LI†	EC‡	Bone	Cartilage	
Adult C57L	C57L	♀	—	3	0	3	3	–	–	–	–	–	–	–	–	2	1				
Adult C57L	C57L	♂♀	—	11	0	14	11	–	–	–	–	–	–	–	–	10	7	—	3		
Adult C57L	C57L	♂♀	DBA§	4	0	16	4	–	–	–	–	–	–	–	–	4	3	—	—	—	Reticulum-cell neoplasm B
Adult A	A	♂♀	DBA	18	2	9	18	–	–	–	–	–	–	–	–	14	15	—	2	—	Squamous-cell carcinoma
		♂♀	—	8	4	13	8	–	–	–	–	–	–	–	–	5	8	—	—	—	Dermal-appendage tumor
		♂	—	8	5	13	8	–	–	–	–	–	–	–	–	6	7				
Adult A	A	♀	DBA	11	11	13	6	4	–	1	–	–	–	–	–	4	8	—	—	—	2 Squamous-cell carcinomas, skin, 3 granulosa-cell tumors, 1 papilloma—skin, 1 dermal-appendage tumor
Fetal C57L	C57L	♂♀	DBA	12	12	11	6	6	–	–	–	–	–	–	–	10	7	—	2	—	Fibrosarcoma, 2 reticulum-cell neoplasms A
		♂♀	—	7	0	10	7	–	–	–	–	–	–	–	–	3	6	1	1	2	Reticulum-cell neoplasm B
Fetal C57L	C57L	♂♂	—	7	0	6	7	–	–	–	–	–	–	–	–	1	5	2	—	2	
		♀	DBA	6	3	18	6	–	–	–	–	–	–	–	–	3	4	1	1	1	Reticulum-cell neoplasm B, mammary tumor (modified C)
		♂♀	DBA	5	3	16	5	–	–	–	–	–	–	–	–	1	4	—	3	—	Hepatoma
Fetal A	A	♂♀	—	6	1	13	6	–	–	–	–	–	–	–	–	2	5	2	3	1	Granulosa-cell tumor
		Intersex	—	1	0	9	1	–	–	–	–	–	–	–	–	—	—	1	—	1	
		♂♀	—	7	2	13	6	1	–	–	–	–	–	–	–	4	5	3	1	3	Bronchial adenoma in transplant
Fetal A	A	♂♀	DBA	6	6	14	0	–	1	–	–	2	2	1	–	2	4	2	—	2	Myoepithelioma, pulmonary adenomatosis in transplant
		♂♀	DBA	8	8	15	0	1	1	5	–	–	1	–	–	2	7	—	—	—	
Adult C57L	LA F_1	♂♀♂	—	8	0	22	8	–	–	–	–	–	–	–	–	7	8	—	1	1	3 Reticulum-cell neoplasms A, hepatoma
		♂	—	8	0	12	8	–	–	–	–	–	–	–	–	8	7	—	3	—	Reticulum-cell neoplasm B
Adult C57L	LA F_1	♀	DBA	11	11	20	10	1	–	–	–	–	–	–	–	9	11	—	—	—	Mast-cell tumor, squamous-cell carcinoma of forestomach with ovarian metastases, dermal-appendage tumor, squamous-cell carcinoma—skin, reticulum-cell neoplasm A, mammary tumor C
		♂	DBA	14	12	12	14	–	–	–	–	–	–	–	–	10	13	—	1	—	Lymphosarcoma
Adult A	LA F_1	♂♀	—	8	1	13	8	–	–	–	–	–	–	–	–	5	7	1	—	—	2 Papillomas of skin
		♂	—	6	1	12	5	1	–	–	–	–	–	–	–	5	5				
Adult A	LA F_1	♀	DBA	8	8	16	6	1	1	–	–	–	–	–	–	4	4	—	—	—	2 Reticulum-cell neoplasms A, squamous-cell carcinoma—forestomach, mammary tumor B, lymphocytic neoplasm (unclassified)
		♂	DBA	11	11	13	5	4	2	–	–	–	–	–	–	7	9	—	1	—	2 Lymphosarcomas, skin papilloma (squamous)
Fetal C57L	LA F_1	♀	—	6	2	23	5	1	–	–	–	–	–	–	–	5	6	1	3	—	Harderian-gland tumor, reticulum-cell neoplasm A
		♂♀	—	7	3	9	7	–	–	–	–	–	–	–	–	5	6	2	1		
Fetal C57L	LA F_1	♂♀♂	DBA	8	8	19	7	1	–	–	–	–	–	–	–	5	7	—	3	1	Melanotic lesion, reticulum-cell neoplasm B
		♂♀	DBA	5	5	15	5	–	–	–	–	–	–	–	–	1	5	—	4	—	Hepatoma
Fetal A	LA F_1	♂♀	—	7	4	21	5	2	–	–	–	–	–	–	–	3	5	4	2	1	Reticulum-cell neoplasm A, reticulum-cell neoplasm B, papilloma
		♂	—	7	2	14	6	1	–	–	–	–	–	–	–	2	6	3	3	5	Harderian-gland tumor
Fetal A	LA F_1	♂♀	DBA	8	8	18	–	2	1	1	1	1	–	1	1	2	7	—	—	1	Hepatoma

Adult C57L	AL F_1	♂♀♂	DBA	7	7	17	1	2	1	1	2	–	–	–	–	1	6	1	1	2	Fibrosarcoma, hepatoma
			—	8	1	19	8	–	–	–	–	–	–	–	–	7	6	1	—	—	Papilloma, reticulum-cell neoplasm B
			—	7	3	15	7	–	–	–	–	–	–	–	–	6	7	—	1	1	
Adult C57L	AL F_1	♀	DBA	10	10	16	10	–	–	–	–	–	–	–	–	9	10	—	—	—	Papilloma, mammary tumor B, granulosa-cell tumor, reticulum-cell neoplasm B, dermal-appendage tumor
		♂	DBA	9	9	12	9	–	–	–	–	–	–	–	–	5	6	—	1	—	Hemangioendothelioma
Adult A	AL F_1	♀	—	8	4	20	8	–	–	–	–	–	–	–	–	4	4	—	—	—	Reticulum-cell sarcoma, reticulum-cell neoplasm B, dermal-appendage tumor, papilloma
		♂	—	7	2	13	7	–	–	–	–	–	–	–	–	5	6	—	—	1	
Adult A	AL F_1	♀	DBA	7	7	14	4	2	1	–	–	–	–	–	–	2	3	—	1	1	Reticulum-cell neoplasm A, mammary tumor B, granulosa-cell tumor metastatic to lung
Fetal C57L	AL F_1	♂♀	DBA	8	8	11	7	–	1	–	–	–	–	–	–	5	6	—	—	—	Lymphosarcoma, squamous papilloma
			—	6	1	21	6	–	–	–	–	–	–	–	–	2	6	2	3	—	Mammary tumor B
Fetal A	AL F_1	♂♀♂	—	7	0	10	7	–	–	–	–	–	–	–	–	1	4	1	2	2	
		♂	—	8	4	18	8	–	–	–	–	–	–	–	–	4	5	4	4	4	
																2	8	—	5	2	Hepatoma

*BP = Bronchial proliferation.
†LI = Lymphocytic infiltration.
‡EC = Epidermoid cysts.
§DBA = Dibenz[*a*,*h*]anthracene.
‖One appeared to be primary in the transplant.

transplants with no recognizable extrapulmonary structures and are interpreted as representing squamous metaplasia of bronchial epithelium. In this situation one is forced to conclude that the fetal bronchus is more susceptible to metaplasia than is the transplanted bronchus of adult lung. Another feature of this lesion, however, is not so readily explained. Only 4 instances of epidermoid cysts were seen in transplants in hosts that received carcinogen. The remainder occurred in host animals without such injections. This certainly suggests that the carcinogen is not responsible for the metaplastic changes and may indeed counteract agents that produce this metaplasia.

The presence of bone was somewhat more common in the fetal transplants than in the adult. The origin of this bone remains obscure. In rare instances, the bone appeared in a fragment of transplanted myocardium. In most animals, however, it was present as an isolated spicule surrounded by otherwise unremarkable transplanted tissue. The cartilage present in transplants probably represents normal bronchial cartilage.

Two other lesions observed in transplanted lungs deserve comment. In 1 fetal A transplant, in an injected strain A host, there was an area of pulmonary adenomatosis that would fulfill the criteria for this diagnosis established by Horn and her collaborators (*8*). Since it was found in only 1 transplant, no conclusions can be drawn regarding etiologic factors and it must be considered as simply an incidental finding. One other transplant, a fetal A transplant in a noninjected A host, contained a lesion that we have interpreted as a bronchial adenoma (fig. 1). It was present in 4 of the serial slides and consisted of a papillary growth into the bronchial lumen. Some of the epithelial cells were ciliated. Periodic acid-Schiff stains on this lesion were negative. It is different from the ordinary adenomatous lung tumor and certainly more papillary than the usual area of bronchial proliferation.

Four tumors, which had reached considerable size in the fetal A lung transplants, were transplanted. One of the 4 occurred in a transplant in a strain A host; the other 3 were found in transplants in LA F_1 hosts. The tumor from the strain A host transplant has now been carried through 32 transplant generations in strain A mice. This tumor has not shown the papillary form so often seen in large lung tumors but has shown a more solid form, with fibrous areas appearing in certain of the transplants. Two tumors from transplants in LA F_1 hosts have been carried through 24 and 84 transplant generations in strain A mice. First-generation transplants also grew successfully in LA F_1 hosts, as was to be expected, and later transplants were made only in strain A hosts. Both of these tumors maintained the histologic appearance of the original tumor. A third tumor, arising in a fetal A transplant in an LA F_1 host, has been successfully transplanted through 80 generations in mice of the donor strain A. Again, first-generation transplants also grew successfully in LA F_1 hosts. The original sections of this tumor show sarcomatous areas (figs. 3–6) and during transplantation the tumor has changed completely to a sarcomatous tumor similar to those described by Stewart (*9*).

The fact that these tumors arising in the A transplants in LA F_1 hosts were transplantable back to strain A mice offers evidence that they were derived from the transplanted A lung. This is of special interest in the tumor with sarcomatous degeneration and suggests that in this condition the sarcoma cells are derived from the transplanted pulmonary tissue and not from stroma of the host. A more detailed study of this phenomenon in another series of transplants is nearing completion (*10*).

Occurrence of Pulmonary Tumors in the Lung Transplants and in the Lungs of the Hosts

Tumors in the lung transplants and in the hosts are listed in table 1.

In all groups, strain C57L lung remained resistant to the development of tumors following transplantation into other resistant strain C57L hosts or into susceptible F_1 hosts, not only when the adult lung was transplanted but also when the fetal lung was transplanted. Tumors were not observed in any of the C57L fetal or adult lung transplants in C57L hosts, in groups injected with the carcinogen or in the noninjected groups, or in any of the adult C57L lung transplants in either LA F_1 or AL F_1 hosts that were not injected. However, 1 pulmonary tumor was observed in an adult C57L lung transplant in an injected LA F_1 host, 1 in a fetal C57L lung transplant in an injected LA F_1 host, and 1 in a fetal C57L lung transplant in a noninjected LA F_1 host.

None of the noninjected strain C57L hosts had tumors in their own lungs, although a few tumors occurred in the lungs of injected C57L hosts. However, in those with tumors there was usually only 1 nodule, indicating the high degree of genetic resistance of the strain. The fact that some of the C57L hosts had tumors in their own lungs, whereas tumors did not occur in the transplants they were carrying, can probably be explained merely on the basis of difference in amount of lung tissue even when one considers the certain amount of growth of the fetal transplants following implantation.

The higher degree of genetic susceptibility of the LA F_1 and AL F_1 hosts is shown by the occurrence of tumors in the lungs of a number of the noninjected F_1 hosts and in most of those injected with the carcinogen. In the injected F_1 hosts with tumors, multiple nodules were observed.

Strain A lung remained susceptible following transplantation into either strain A or F_1 hosts. This was particularly evident in fetal lung transplants in injected hosts and also, to a lesser degree, in adult lung transplants in injected hosts.

Tumors occurred in 1 or both fetal A lung transplants in all injected A hosts, with an average of 3.9 tumors for the 2 transplants in each host, and in all but 1 of the injected LA F_1 hosts, with an average of 3.1 tumors for the 2 transplants in each host. Tumors occurred in 1 or both adult A lung transplants in 48 percent of the injected A hosts, in 42 percent of the injected LA F_1 hosts, and in 27 percent of the AL F_1 hosts. In these noninjected hosts there was usually only 1 nodule in the 2 transplants, and no animal had more than 3 nodules.

In adult A lung transplants in noninjected A hosts, no tumors occurred, but tumors occurred in fetal A transplants in 2 noninjected A hosts. Of the noninjected F_1 hosts bearing adult A lung transplants, 1 LA F_1 host had a tumor in its transplant, but none of the AL F_1 hosts had transplants with tumors. Of those bearing fetal A lung transplants, 3 LA F_1 hosts had transplants with tumors, and, again, no AL F_1 hosts had transplants with tumors.

Some of the noninjected A and F_1 hosts of A lung transplants had tumors in their own lungs, and all the injected A and all but 1 of the injected F_1 hosts had multiple tumors in their own lungs.

Discussion

The data presented confirm the earlier observations that the genetic susceptibility of the lungs of strain A mice to tumors induced by dibenz-[*a*,*h*]anthracene and the genetic resistance of the lungs of strain C57L mice are established in young adults and are not reversed by transplantation into LA F_1 hosts, whose genetic susceptibility is less than that of strain A and greater than that of strain C57L. These observations are extended to enable one to make comparisons between results with (*a*) young adult lung transplants and those of fetal lung transplants, (*b*) tumors induced with dibenz[*a*,*h*]anthracene and spontaneous tumors, (*c*) transplants in LA F_1 hybrids and those in their reciprocal AL F_1 hybrids, and (*d*) transplants in the strain of origin and those in the F_1 hosts.

The primary difference between the fetal lung transplants and the young adult transplants, both of which were placed in young adult hosts, was the fact that the fetal transplants grew, whereas the young adult lung transplants remained viable without any increase in size except that due to the neoplasm. Each fetal transplant was a complete lobe of the lung of the fetus and, although each was very small when transplanted, it continued to grow until in many instances it had reached a size approximating that of an adult lung lobe. More induced tumors occurred in such transplants than in the adult lung transplants, but the number was no greater in proportion to the size of the transplant.

Just as strain C57L adult lung transplants remained resistant and strain A adult lung transplants remained susceptible when transplanted into genetically identical F_1 hosts, so did the strain C57L fetal lung transplants remain resistant and the strain A fetal lung transplants remain susceptible when transplanted into the F_1 hosts. Thus the genetic susceptibility and resistance that is fixed in adult lung and not altered by a genetically different host is irreversibly fixed as early as fetal development.

The effect of the carcinogen on the occurrence of lung tumors was as clearly shown in the lung transplants as in the lungs of the hosts. Although tumor response was marked in susceptible transplants in injected hosts, particularly in the fetal transplants where multiple tumors occurred, only 3 tumors occurred in transplants in noninjected hosts. As could have been expected, all 3 were in strain A transplants, 1 in an adult and 2 in fetal transplants.

No significant difference was observed in comparable transplants in reciprocal hybrids. Although the study lacked a group of injected AL F_1 hosts with fetal A lung transplants—an important group for comparison with injected LA F_1 hosts with such transplants—the occurrence of tumors in adult A transplants in injected AL F_1 hosts was not significantly different from that in adult A transplants in injected LA F_1 hosts.

There is an indication of a slight influence by the host on the susceptibility of the transplant. The average number of tumors in the fetal A transplants in injected A hosts was slightly greater than that in fetal A transplants in injected LA F_1 hosts, and the incidence of tumors in adult A transplants in injected A hosts was slightly greater than that in adult A transplants in either LA F_1 or AL F_1 injected hosts. Furthermore, the 3 tumors occurring in strain C57L transplants were in F_1 hosts. The number in the comparable groups is small, and the differences cannot be considered highly significant, but they are in agreement with observations made in the earlier study (*1*).

The isologous transplants, furthermore, offered an opportunity to see whether transplantation *per se* had any influence upon tumor occurrence. When the size of the transplants was considered, the occurrence of tumors in the transplants was comparable with that in the intact lung, indicating no discernible effect of the transplantation *per se* on the occurrence of the tumors.

Summary

The observation was confirmed that most of the difference in degree of genetic susceptibility to tumor formation, in susceptible strain A and resistant strain C57L lung, is so fixed in adult lung that it is not altered when transplanted to the same LA F_1 hosts. This observation was extended to fetal lung, and it was found that even in fetal development the difference in genetic susceptibility was likewise irreversibly fixed.

More tumors occurred in the fetal lung transplants than in the adult lung transplants. However, following transplantation, the fetal lung transplants grew and became considerably larger than the adult lung transplants.

Transplantation *per se* did not appear to increase or decrease the occurrence of tumors in the lung transplants.

No difference was apparent between reciprocal F_1 hosts with respect to the occurrence of tumors in the transplants they carried.

Fewer tumors occurred in A transplants in F_1 hosts than in A transplants in A hosts. The 3 tumors observed in C57L transplants were all in F_1 hosts; none were in C57L hosts. There were few animals in comparable groups, and the possible influence of the host on susceptibility of the transplants may not be significant.

Tumors that occurred in 4 of the fetal A lung transplants, 3 in injected F_1 hosts and 1 in injected A hosts, grew to considerable size and have been transplanted successfully in the original donor strain A through many generations. One of these tumors from a strain A transplant in an F_1 host, in the original tumor, showed sarcomatous elements that became

predominant in later transplant generations in strain A. Thus, the sarcomatous elements must have originated in the A lung transplant and not from cells of the F_1 host.

Other lesions that occurred rather frequently in the transplants included bronchial proliferations, lymphocytic infiltration, and epidermoid cysts. The frequency of occurrence of bronchial proliferation was higher in the transplants of strain C57L lung than in those of strain A lung and higher in the transplants of adult lung than in those of fetal lung. The epidermoid cysts occurred much more frequently in the transplants of fetal lung than in those of adult lung. The presence of bone was also noted in a number of the transplants and was more frequent in the transplants of fetal than adult lung. Cartilage was also noted in a number of the transplants. Other lesions that occurred less frequently in the transplants and in the hosts were recorded.

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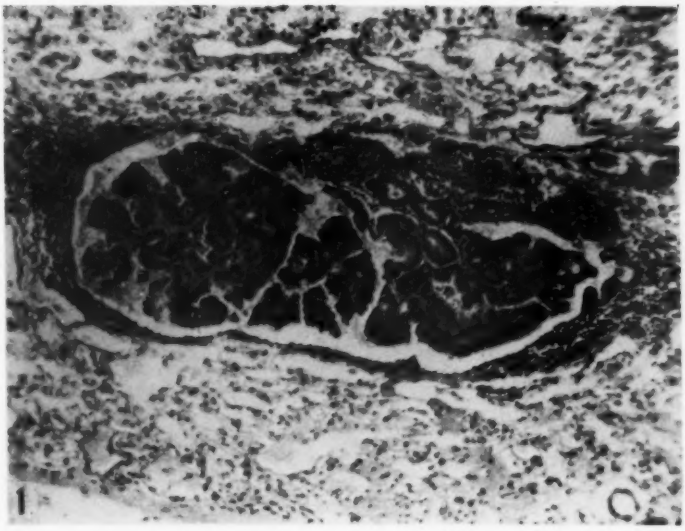
Plate 67

Figure 1.—Bronchial adenoma (fetal A transplant in noninjected A host). × 100

Figure 2.—Bronchial proliferation, showing continuity of epithelium between a bronchiole and neighboring alveolar spaces. × 200



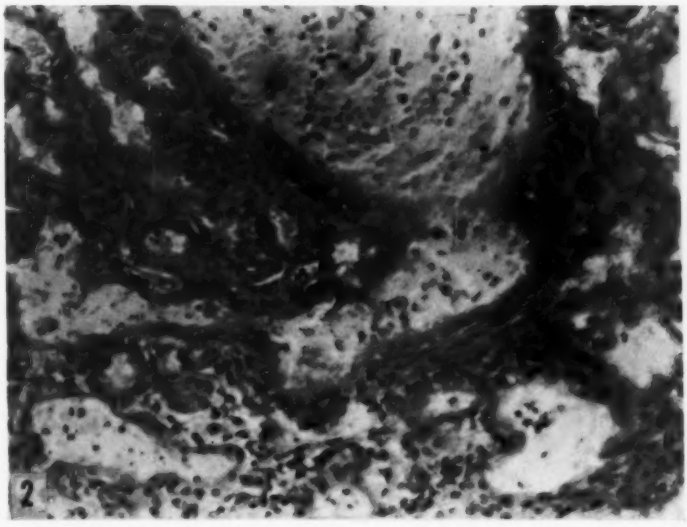


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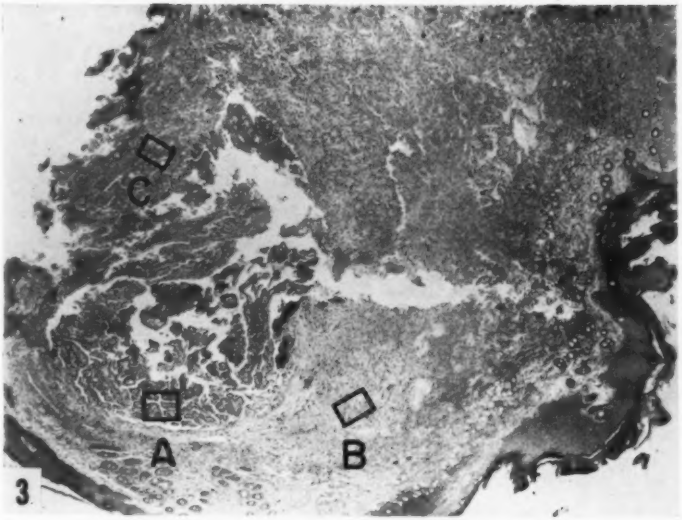
PLATE 68

FIGURE 3.—First-generation transplant of a tumor that ultimately became sarcomatous. This tumor shows papillary and solid carcinomatous areas and an area of sarcomatous transformation. × 20

FIGURE 4.—Papillary region of tumor shown in A of figure 3. × 516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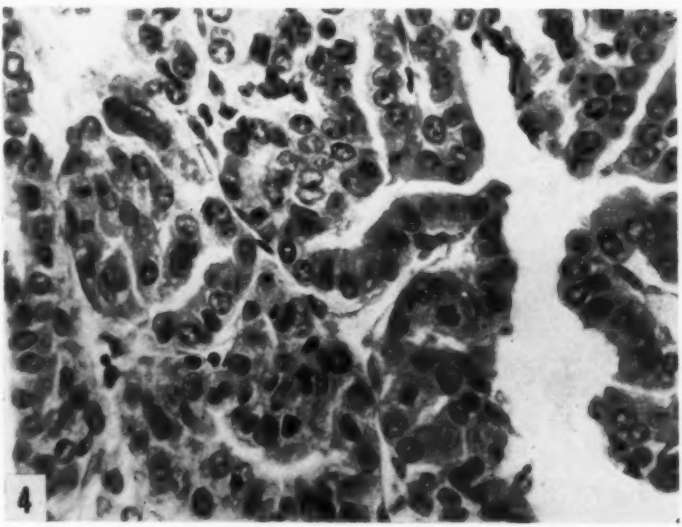
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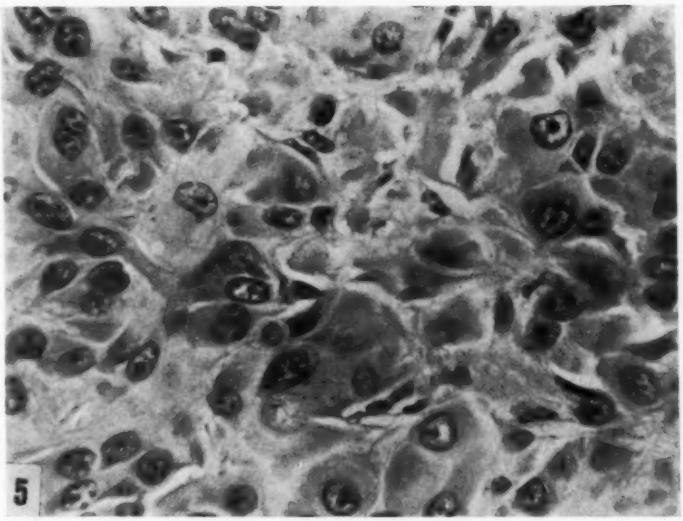
PLATE 69

FIGURE 5.—Solid carcinomatous area of tumor shown in B of figure 3. × 516

FIGURE 6.—Sarcomatous area of tumor shown in C of figure 3. × 516



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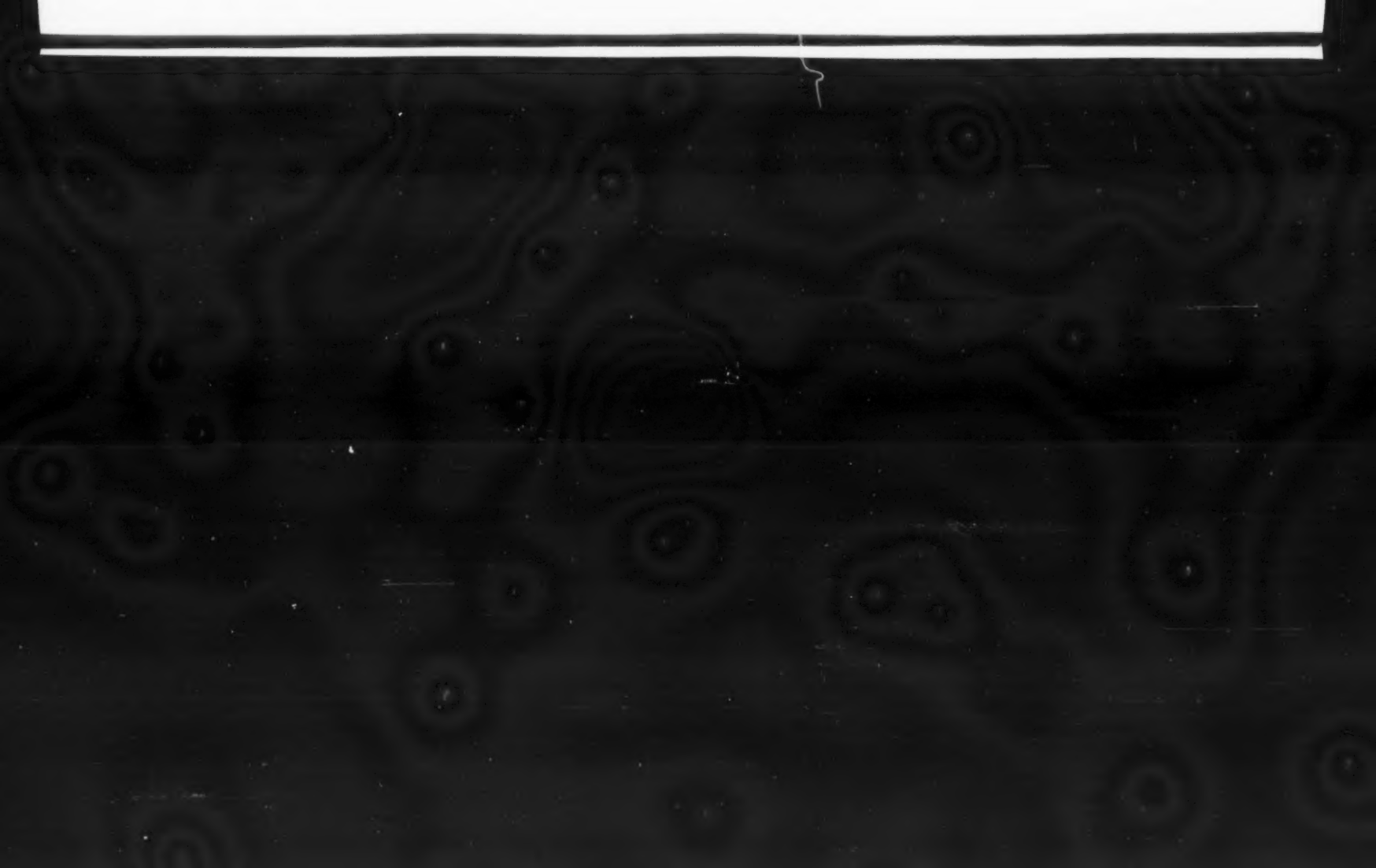

The Secretory and Clinical Aspects of Achlorhydria and Gastric Atrophy as Precursors of Gastric Cancer [1,2,3]

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Present knowledge of malignancies of the stomach suggests that gastric cancer is associated with anacidity and gastric mucosal atrophy. In recent decades there has been a voluminous literature on the subject that either supports or opposes the significance of this association. In our investigations, my colleagues and I have been unable to demonstrate a causal relationship between atrophy of the gastric mucosa and gastric carcinoma (*1–7*). However, a critical evaluation of results from our laboratory and clinical experiments performed during the past decade confirm the usefulness of the relationship in the early diagnosis of gastric carcinoma in asymptomatic patients (*8–12*). Earlier diagnosis should increase the survival rate of patients with gastric cancer. Further, we believe a large body of corollary evidence supports the concept of a close, though unexplained, association between atrophy of the gastric mucosa and gastric cancer (*13–18*). How and why gastric cells become malignant is not known and the answer depends on progress in further investigations.

Achlorhydria and Gastric Atrophy

Most stomachs that are achlorhydric, after stimulation with triple histamine, show a diffuse atrophy of the mucosa. Hebbel (*19*) has demonstrated a severe or moderate degree of mucosal atrophy in 72 percent of such stomachs (table 1). This represents histomorphologic change commensurate with anacidity, on the basis of the absence of parietal cells. In our experience, however, 4.5 percent of stomachs that are achlorhydric after stimulation with triple histamine have a histologically normal gastric mucosa in the region of the antrum and the body.

In Hebbel's experience, stomachs that secrete more than a few degrees of free hydrochloric acid never show a severe degree of mucosal atrophy. He has found moderate or mild mucosal atrophy in 17 percent of 63

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[3] Presented to the New York Academy of Gastroenterology, April 9, 1956.

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stomachs that were hypochlorhydric or normochlorhydric prior to surgery. All his specimens had a carcinoma in a portion of the subtotally resected stomach.

In a group of 8 patients (table 2) collected by Dr. MacLean, 2 to 10 years after subtotal gastrectomy of Billroth II type, all developed clinical pernicious anemia; all 8 specimens had severe and diffuse gastric mucosal atrophy on histologic examination of the resected specimen (*20*). Three patients had a Billroth II type of resection for gastric ulcer and had normal levels of free hydrochloric acid preoperatively. The findings in these 3 cases contrast with those of Hebbel. In these instances, it may be postulated that parietal cells functioned in the fundus and body of the stomach remaining as the postresection pouch, and, subsequently, the mucosa became achylic and pernicious anemia resulted.

After reviewing surgically removed specimens, it seems clear that a functional type of achlorhydria (in the presence of histamine stimulation)

TABLE 1.—*Mucosal changes related to gastric acidity**†

	Normal mucosa	Partial atrophy	Diffuse atrophy				Total	Percent
			Total	Severe	Moderate	Mild		
Achlorhydria	5	15	90‡	53	27	10	110	63
Hypochlorhydria (less than 20°)	5	5	7	0	6	1	17	10
Free acid over 20°	22	20	4	0	3	1	46	27
No. gastric cancers							173	
Total with atrophy								81
Diffuse atrophy								58
Partial atrophy								23

*Data from Hebbel (*19*).
†In all cases, acidity was determined with histamine.
‡Of this group, the severe and moderate cases combined comprised 72 percent.

does exist in at least a small percentage of stomachs with a carcinoma. Whether there is any difference in the cancer susceptibility of an anacidic stomach with a histologically normal mucosa, as compared to an anacidic stomach with demonstrable diffuse mucosal atrophy, cannot be categorically stated. However, experience with pernicious-anemia patients and with others with gastric polyps would lead us to postulate greater susceptibility to cancer in stomachs with diffuse and severe mucosal atrophy (*21–35*). The appearance of gastric cancer in the stomachs of pernicious-anemia patients, in whom severe gastric atrophy is always present, is 21.3 times more frequent than in a similar group of normal people (*10*).

In our clinic, when a biopsy is taken with the Benedict operating gastroscope, normal appearance of the mucosa of the stomach is confirmed histologically in only 54 percent of the cases. When a gastroscopic diagnosis of atrophic mucosa is made, we have found histologic

TABLE 2.—*Patients with subtotal gastrectomy and subsequent pernicious anemia**

Patient	Age at operation	Preoperative diagnosis (confirmed)	Date of gastrectomy	Type of resection	Free acid preoperatively (*degrees*)	Date patient was diagnosed	Bone marrow	Neurologic findings
RF	60	Gastric ulcer	4–18–49	80% B II	38	6– 1–54	Megaloblastic	Present
AT	80	Carcinoma	3–15–48	80% B II	0	2– 3–54	Megaloblastic	None
EB	61	Gastric ulcer	12–16–40	90% B II	38	8– 1–47	Megaloblastic	Present
AW	72	Carcinoma	12– 1–47	75% B II	—	10–12–52	Megaloblastic (on smear)	None
JH	53	Gastric ulcer	3–13–42	80% B II	40	7– 7–52	Megaloblastic	None
IO	57	Carcinoma	3–23–45	90% B II	0	1–30–51	Megaloblastic	None
WH	60	Carcinoma	8–28–44	75% B II	0	12–30–49	Megaloblastic	None
EJ	23	Polyps	9–10–45	80% B II	0	11– 3–47	Megaloblastic	None

*All of these specimens had diffuse, severe gastric atrophy on histologic examination.

confirmation in 74 percent of the cases. The diagnosis of hypertrophic mucosa has not been supported histologically in any of our cases (*36*).

Strict reliance upon gastroscopic diagnosis of the condition of the mucosa is not possible at this time, but the addition of multiple biopsies, from various areas of the stomach, increases the usefulness of this examination. With the biopsy technique, the patchy distribution of mucosal atrophy, reported by Hebbel in surgical specimens, has been confirmed by Ylvisaker *et al.* (*36*). In a group of achlorhydric patients without demonstrable cancer, the mucosa was histologically verified as atrophic in 78 percent; the remaining 22 percent had a normal mucosa insofar as gastroscopic biopsies may be considered a reliable means of confirmation. Again, the evidence would indicate a functional achlorhydria, since histamine stimulation had failed to initiate acid secretion in these mucosae, which contained apparently normal parietal cells (*36*).

In 1949, Cromer, Comfort, and Butt studied 185 patients with proved adenomatous gastric polyps and found the incidence of achlorhydria to be greater by 65 percent than would be expected in a group of normal persons of the same age and sex. They found, by comparison, that the percentage of achlorhydria approached that of adenomatous polyp-bearing stomachs, namely, 90 percent or more, only when gastric cancers were about 7×7 cm. in size. Their resectable gastric-cancer patients had achlorhydria in only 51 percent of cases. Patients with gastric cancers less than 3 cm. in diameter showed only 18 percent achlorhydria. In these 185 polyp-bearing stomachs, cancer was present in 73 instances (40%) either in the polyp or as a separate lesion; 19 of the 185 people had pernicious anemia, and among these 9 cancers were found (47%) (*22*).

In our experience at the University of Minnesota Hospitals, the association of gastric atrophy with true adenomatous polyps (in surgically removed stomachs) has been over 93 percent (*25*, *37*). It is difficult not to relate causally the high percentage of malignancy in such instances (47% when adenomas exceed 2 cm. in diameter) with the unusually high association of mucosal atrophy (93%) and achlorhydria (98%), as seen in our material.

In the studies of Cromer *et al.*, stimulation for hydrochloric acid was a test meal of 8 arrowroot cookies and 400 cc. of water (*22*). At the University of Minnesota, during the last decade, we have routinely used triple-histamine stimulation of gastric acid (0.5 mg. per dose spaced at half-hour intervals) and have noted achlorhydria or hypochlorhydria in 87.8 percent of our 1,152 gastric-cancer patients. We believe this vigorous method of parietal-cell stimulation more truly exhausts the mucosa and more accurately represents the state of secretory activity of the mucosa (*10*).

Recently, we have been using a modification of the augmented histamine test, as described by McKay (*38*), in which the parenteral antihistaminic phenylpyramine (Histadyl, Lilly) is given before the test to allay side effects. One-half hour later an augmented dose of histamine (0.1 mg. per 10 kg. of body weight) is given the patient. If no free acid is present after the first dose of histamine, the same dose is repeated, and

another examination for free acid is made. If there is still no free acid, the same dose is repeated a third time. Using this method, we have avoided a great many of the disagreeable side effects of large doses of histamine. Five percent of the known achlorhydric persons have been shown to have some free acid (*10*).

Most observers agree that gastric mucosal atrophy is relatively common in older people (*17, 39*). Hebbel found that 72 percent of stomachs, from individuals of all ages, contained some degree of atrophic gastritis. In a group of people over 50 years of age without malignancy, he found atrophy, comparable to that seen in carcinoma-bearing stomachs, in the antrum in 13 percent and in the body in 20 percent. General patterns of distribution of atrophic gastritic changes have been found to be the same in stomachs with or without cancer (*19*). It seems well established that gastric mucosal atrophy of a mild or patchy degree can be found in stomachs of well persons, occasional patients with duodenal ulcer, and patients with extragastric malignancy. From clinical material it can be concluded that a much higher correlation of severe gastric mucosal atrophy is found in persons with gastric cancer than in any other group, except pernicious-anemia patients. On the other hand, gastric carcinoma can exist in a stomach having all the histologic features of normalcy and a normal secretory capacity.

We have never been aware of a demonstrable qualitative difference between carcinomas found under these varied circumstances.

Relationship of Lower Animals to Man with Respect to Gastric Atrophy and Cancer

The spontaneous appearance of adenocarcinomas of the stomach in lower animals is an extremely rare phenomenon. Up to 1936, only 3 cases of spontaneous gastric adenocarcinoma in mice had been reported in the literature, and within recent years only a few additional cancers have been found among thousands of mice autopsied (*1, 4*).

Similarly, among other animals, both domestic and wild, spontaneous cancer of the stomach occurs only rarely. Jackson, writing in 1936 on equine tumors, described only 1 case of gastric carcinoma among 119 malignant lesions. In describing various cancers in other domestic animals, he was unable to report additional gastric carcinomas.

The stomachs of such animals as mice, dogs, cats, rabbits, hamsters, and monkeys have resisted, almost completely, numerous and varied attempts to induce gastric malignancy. Ivy and coworkers in 1950 produced ulceration in the stomachs of rabbits by vagotomy or by excision of areas of the mucosa. They failed to induce adenocarcinomas by prolonged exposure of the ulcers to methylcholanthrene.

Direct implantation of potent carcinogens into the walls of the stomach of rodents has produced a small percentage of true adenocarcinomas (*2, 3*) of that organ. Thus, it appears that gastric glandular cells of some lower animals, at least mice, probably do not possess a complete species resistance to neoplastic change or an immunity to the action of

certain chemical carcinogens; rather, protective mechanisms may be present which negate the action of carcinogens given as dietary supplements. Or it may be that *certain predisposing factors or conditions found in man are lacking in lower animals* (*4*).

The human stomach differs from the animal stomach in several specific features. Atrophic gastritis, either as a disease in itself or in connection with pernicious anemia, is prevalent among human beings; in lower animals not only does it fail to develop spontaneously but also it is impossible to produce atrophic gastritis of a permanent type in any laboratory animal. Gastric ulcers are a common medical problem in man, but in lower animals gastric mucosal ulceration can be produced and maintained only by drastic experimental procedures, such as excision of the gastric mucosa or tying the vagus nerves in the rabbit, induction of severe calcium deficiency in rats, or exposure of the gastric mucosa of rats and mice to radiations of roentgen rays or radioactive isotopes.

In addition, gastric polyps are an important precancerous lesion in the human stomach, but they do not occur spontaneously in animals. Monkeys exposed to vapors from shale oil have developed polypoid lesions of the gastric mucosa, but, as far as we know at the present time, these are not permanent. The resistance of the stomachs of animals to the changes of atrophic gastritis seems to be an important factor in their freedom from gastric cancer.

During recent years, one of us (C.R.H.) has tried to overcome possible protective mechanisms preventing the induction of adenocarcinomas of the mouse stomach by oral administration of methylcholanthrene. In order to abet the action of methylcholanthrene, the carcinogen was given in combination with the following: whole bile salts; lysozyme; eugenol and heat as chemical-physical desquamators; diets deficient in vitamins A and B or proteins; exposure of the gastric mucosa to emanations from radioactive phosphorus (*2–5*).

No adenocarcinomas developed from these experiments, but in the stomachs of mice exposed to irradiation through the medium of radiophosphorus, mucosal atrophy resulted which resembled that found in man (*5*). The uniform absence of gastric mucosal atrophy, megaloblastic anemia (pernicious anemia), adenomatous gastric polyps, and gastric cancer in lower animals contrasts strongly with the situation in man. The frequent coexistence of achlorhydria, gastric adenomas, mucosal atrophy, pernicious anemia, and carcinoma of the stomach, in one individual, compels one to consider an etiologic relationship.

Achlorhydria as a Screening Test for Gastric Cancer: Preliminary Study (Study A)

Perhaps the most convincing evidence of a possible causal relationship between achlorhydria and gastric cancer would be the demonstration of a significantly greater incidence of the disease in a large number of achlorhydric persons observed for a prolonged period of time compared with a decreased incidence in a similar group of normochlorhydric people.

Furthermore, more favorable end results for the achlorhydric group than for the general population with respect to early and accurate diagnosis would give added support for the use of achlorhydria as a screening test in large segments of the population.

Starting with this premise, attempts have been made at the University of Minnesota to diagnose gastric cancer in the *asymptomatic stage* of the disease. The problem has been approached through annual or semiannual roentgenologic examinations of certain groups of people believed to have a significantly increased tendency to develop gastric cancer (*10, 12, 40–42*).

During a 10½-year study at the University of Minnesota, over 12,700 men and women have been studied in the achlorhydria-hypochlorhydria screening tests for persons apt to develop gastric cancer. The groups in the initial project comprised persons with (*a*) pernicious anemia, (*b*) achlorhydria and hypochlorhydria, and (*c*) a family history of gastric cancer. Since none of the blood relatives of gastric-cancer patients developed gastric cancer over a 7½-year period, they were dropped from the study (*10*).

At the start of study A, outpatient registrants, over 50 years of age, in the University Hospital clinics were tested for gastric hydrochloric-acid secretion by the intubation method. Three injections of histamine diphosphate were given to stimulate the gastric mucosa. Examinees with complete absence of free hydrochloric acid after triple-histamine stimulation were designated "achlorhydric," and those with less than 30 degrees of free acid were considered "hypochlorhydric." Initially, 5,336 gastric expressions were performed, and 1,670 people (31.7%) were found to fit the criteria for this study (table 3).

When possible, 3 successive doses of 0.5 mg. histamine diphosphate were given at 20-minute intervals. If histamine reactions appeared, however, injections were stopped; these examinees were designated separately according to the number of injections given. The standard test, utilizing Töpfer's reagent and 0.1 *N* NaOH, was used throughout the study and proved most satisfactory.

The quininium ion-urinary excretion test (*43*) was tried on several hundred people, but the extraction procedure in the laboratory was too time-consuming. With this technique, results are only qualitative, and since the quantity of free acid is significant, the University of Minnesota group has preferred the intubation-Töpfer's reagent method.

TABLE 3.—*Incidence of achlorhydria and hypochlorhydria in groups of patients over 50 years of age, University of Minnesota Hospitals*

Patients with:	Achlorhydric (*percent*)	Hypochlorhydric—free acid less than 30° (*percent*)
Gastric carcinoma	72. 5	15. 3
Benign extragastric tumors	30. 0	28. 0
Malignant extragastric tumors	33. 0	25. 0
Non-neoplastic conditions	36. 0	23. 0

The examinees were recalled yearly for gastric roentgenograms consisting of fluoroscopic examination after a barium meal. An appropriate number of routine films and spot films were taken. Gastroscopic examinations were not performed routinely but were used primarily for patients with roentgenographic lesions of a questionable nature.

Berkson *et al.*, reporting from the Mayo Clinic in 1952, demonstrated a remarkable correlation between degree of gastric acidity and survival from gastric cancer; the lower the gastric acidity, the poorer the outlook with respect to longevity (*44*).

Pernicious-Anemia Patients

A group of 77 patients with pernicious anemia was studied in a similar manner. In each case, the diagnosis of anemia was established and repeatedly verified by bone-marrow studies.

Incidence of Gastric Cancer in People over 50 Years of Age

On a nation-wide basis, it is difficult to obtain correct incidence values for various forms of cancer. However, if an acceptable and significant evaluation of various screening tests is to be made, we must be able to compare incidence values for a given malignancy in the test group with over-all incidence values for the population at large in the same age group. By using current statistics, the expected yearly incidence of gastric cancer per 100,000 population over 50 years of age has been established. Table 4 demonstrates the step-by-step derivation of the value for gastric cancer, which is 81.2 percent per 100,000 population over 50 years of age.

TABLE 4.—*Incidence of gastric cancer in people over 50 years of age*

Incidence of gastric cancer per 100,000 population per year	22.3% (Levin)*
Percent of gastric cancers occurring over age 50	81.6% (Steiner)†
Percent of total population over age 50	22.4% (U. S. Census)‡
Expected incidence per year per 100,000 population over age 50	81.2%

*New York Cancer Statistics. Albany, N. Y., 1942-1944.
†Cancer, Race and Geography. Baltimore, Williams and Wilkins, 1954, pp. 60-61.
‡U. S. Census, 1950. Washington, D. C., Supt. of Docs., U. S. Government Printing Office.

Cancer Detection Center (Study B)

During the 8 years the Cancer Detection Center at the University of Minnesota has been in operation, a total of 7,375 apparently healthy examinees—3,568 men and 3,807 women—has been screened on the basis of age, achlorhydria, and hypochlorhydria. Because over 80 percent of all gastric cancers occur in persons over 50 years of age, the group tested periodically by gastric roentgenograms was limited to those at this age level, which has reduced the more highly susceptible group to 20 to 25 percent of the over-all population. Since only 25 percent of persons over 50 years of age are achlorhydric or hypochlorhydric, in the experience of the Cancer Detection Center, it was possible to reduce further the size

of the segment of our population that required repeated gastric roentgenograms. *Thus, 6½ percent of the over-all population, or one fourth of the group of persons over 50 years of age, must be studied repeatedly to detect the greatest number of gastric cancers in the asymptomatic stage of the disease.*

The test used for free acid was administration of 1 dose of 0.5 mg. histamine diphosphate as a secretory stimulant. Most of the individuals found to be achlorhydric or hypochlorhydric have been re-examined at yearly intervals by gastric analysis and roentgenograms.

In addition to the achlorhydric group in study B, any examinee with occult blood in the stool, a family history of gastric cancer, or a hemoglobin level below 11.0 gm. had a gastric roentgenogram. *It should be emphasized that even if all other screening techniques except examination for achlorhydria and hypochlorhydria had been omitted, no asymptomatic or symptomatic gastric cancer would have been undetected* (*10*).

Results of Combined Study A (Preliminary Group) and Study B (Cancer Detection Center Group)

We have already published separately the cancer-detection and survival data for study A and study B (*9–11*, *45*, *46*). Since the 2 studies have been in effect for almost an equal length of time—10½ and 8½ years, respectively—the data have been combined for this presentation. In practice, however, these studies have been carried out independently on different groups.

Previously, we emphasized the remarkable uniformity of incidence of gastric cancer in these 2 studies. In the 1955 report, 19 cancers were found in 1,747 achlorhydric or hypochlorhydric patients in study A, and 19 cancers in the 1,769 achlorhydric or hypochlorhydric patients in study B (*10*).

In the hypochlorhydric group of study A, the examinees (271 persons) were found to have a chance of developing gastric cancer 2.6 times greater than that of the normal population of similar age; in the achlorhydric group in study A, that chance was 3.2 times greater. In study B, the achlorhydric and hypochlorhydric groups had 5.2 times as much chance of developing gastric cancer as the normal population (*10*). The length of time each of these studies has been in effect influences the calculations with respect to patient-years of participation.

Tables 5 and 6 show the data for the combined studies as of September 1, 1955. There were 3,439 people in the combined achlorhydric and hypochlorhydric group and 77 pernicious-anemia patients; total in the combined study—3,516. In assembling the group of achlorhydric-hypochlorhydric persons for study, 12,700 persons were screened. In study B, which comprised 7,375 people, 25 percent were placed in this category (*11*).

As shown in table 5, gastric polyps have been found in 2.4 percent of the achlorhydric-hypochlorhydric group (83 patients) and in 7.8 percent of the pernicious-anemia group (6 patients). Carcinomas have been found in 38 of the achlorhydric-hypochlorhydric group (1.1%) and in

TABLE 5.—*Patients with gastric polyps and cancers in the combined studies A and B*

Groups	Number	Polyp cases	Percent	Cancer cases	Percent
Achlorhydric and hypochlorhydric (less than 30° free HCl)	3,439	83	2.4	38	1.1
Pernicious-anemia	77	6	7.8	4	5.2
Total	3,516	89	2.5	42	1.19

4 of the pernicious-anemia group (5.2%). A total of 89 patients have developed gastric polyps (2.5% of 3,516 persons) and 42 patients have developed cancer (1.19% of 3,516 persons).

These figures, converted to number of lesions per year per 100,000 population over 50 years of age, are presented in table 6. The data now permit comparison of the combined incidence of gastric cancer in study A and study B with the established expected incidence of gastric cancer per year per 100,000 of the normal population over 50 years of age. *Table 6 indicates a 4.5 times increase of gastric cancer in the achlorhydric-hypochlorhydric group, and a 21.9 times increase in the pernicious-anemia group. The increase in incidence per year for the entire group of 3,516 persons is 4.9 times greater than in the normal population over 50 years of age, or 490 percent!*

TABLE 6.—*Comparison of gastric-cancer incidence in preliminary and Cancer Detection Center groups with over-all expected yearly incidence in the U. S. for persons over age 50*

Group	Cancers	Incidence per year per 100,000 population over age 50	Increase over expected national incidence*
Achlorhydric and hypochlorhydric (3,439)	38	364	4.5 ×
Pernicious-anemia (77)	4	1,733	21.9 ×
Total (3,516)	42	395	4.9 ×

*Expected annual incidence per 100,000 population over age 50—81.2.

Since 25 percent of the examinees in study B were achlorhydric or hypochlorhydric, we could expect 20 to 25 percent of the general population over 50 years of age to reveal the same results.

The value of this method of population screening has been confirmed by the lack of gastric-cancer development among the 5,532 persons (75%) found to be normochlorhydric in study B. Seventy-six percent of the registrants at the Cancer Detection Center are active in the examination program each year. Known deaths among all registrants total 2 percent. The 22 percent who do not return for an annual examination are circularized by letter or contacted by telephone; we have about 100 percent coverage of this segment of the population. Since the entire examination procedure and method of patient management are the same for all examinees, we

believe the Minnesota Detection Center study, study B, has been well controlled.

The criteria establishing the need for gastric roentgenograms have been achlorhydria-hypochlorhydria or pernicious anemia. In the combined studies of the 42 patients with cancer, 24 had no symptoms at the time of diagnosis. Twelve of the 24 patients are living and well, which shows a survival rate of 50 percent at this point of the study. In the group of 18 patients with symptoms, 7 patients (38%) survived. The over-all survival in the total group of 42 cancer cases is 45 percent for an average of 36 months. Five patients have survived over 5 years, and they were in the asymptomatic group.

The data on gastric cancers in study B are found in table 7.

Retrospective examination of the earlier cancer cases found in the Detection Center revealed that some had had symptoms or signs of disease in the involved organ or organ system. For clarity, we have separated all cancer cases into either asymptomatic or symptomatic types; 10 patients showed no clinical evidence of malignancy at the time the diagnosis was made. Roentgenograms of the stomach were made in all because they were achlorhydric. Only 1 patient had a misleading gastric analysis (low normal) on 1 occasion. Of 20 patients with stomach cancer 19 had achlorhydria. In the asymptomatic group we have 10 percent with local extension, 30 percent with positive lymph nodes, and 70 percent surviving. In the symptomatic group we have 90 percent with local extension, 90 percent with positive lymph nodes, and only 20 percent surviving.

We believe that our studies indicate practical and effective criteria for determining which segments of the total population should be screened, thus reducing to a practical size the group to be examined in screening surveys. Members of this group probably should be examined according to the following schedule:

(*a*) Pernicious anemia patients—gastrointestinal series at 6-month intervals.

(*b*) Achlorhydric-hypochlorhydric persons—gastrointestinal series at 9-to-12-month intervals.

The silent interval of gastric cancer has been found, in our clinic, to average between 18 and 24 months. With a long-range program of yearly

TABLE 7.—*Study B gastric-cancer patients, March 1, 1948 to September 1, 1955*

Group	Asymptomatic 10	Symptomatic 10
Achlorhydric	9	10
Hypochlorhydric	1 (Normochlorhydric on 1 exam) (Misleading)	0
Polypoid-type lesion	5	1
Local extension	1 (10%)	9 (90%)
Positive nodes	3 (30%)	9 (90%)
Survivors	7 (70%)	2 (20%)

re-examination of the more highly susceptible portion of the population, there is reasonable assurance, from our studies, that many more gastric cancers can be detected in the presymptomatic phase of the disease than has heretofore been possible.

Additional Secretory Aspects of the Stomach in Relation to Carcinoma

Possibly an additional etiologic factor in gastric cancer might be a disturbance in the normal production of intrinsic factor. Recently, one of us (L.D.M.), has utilized the Schilling test (urinary excretion of orally administered radioactive vitamin B_{12}) as an assay method for the presence of intrinsic factor in the gastrointestinal tract (*47*, *48*).

Initially in our work, the fecal excretion of orally administered vitamin B_{12} Co^{60} was used as an assay method to detect the presence of intrinsic factor (*47*). Because diarrhea has frequently influenced the results with this method, we now believe it to be inferior to the Schilling test. The urinary-excretion test, in addition to being more accurate, is more easily performed.

An oral dose of 0.37 to 0.9 μg. of vitamin B_{12} Co^{60}, with a specific activity of 770 μC per mg., is administered at 8:00 a.m., after the bladder is emptied. A flushing dose of 1000 μg. of nonradioactive vitamin B_{12} is immediately given intramuscularly. The urine is collected for 24 hours, and ten 5 cc. aliquots are examined for radioactivity; the total 24-hour output of radioactivity is calculated and expressed as a percentage of the oral dose.

With this test, the range of excretion in normal persons was between 6.7 and 31 percent of the administered oral dose. While the test is not quantitative at the present time, the variation in values may be indicative of differences in the quantity of intrinsic factor produced and may be used for this purpose in the future.

In contrast, patients with pernicious anemia and those with total gastrectomy do not excrete radioactive vitamin B_{12} in the urine in detectable amounts, which indicates virtually a complete lack of absorption of radioactive vitamin B_{12} (extrinsic factor) by the gastrointestinal tract.

More important, a recent study of 55 patients with histamine-fast achlorhydria, but without clinical evidence of pernicious anemia, has revealed failure, in 2 patients, to excrete any of the orally administered radioactive vitamin B_{12}. The addition of oral intrinsic factor with the radioactive vitamin B_{12} resulted in normal absorption from the gastrointestinal tract and excretion of normal amounts of radioactive material in the urine (*49*).

We believe that patients with achylia gastrica, demonstrated in this manner, probably have the same predilection for gastric cancer as those with clinically evident pernicious anemia. The importance of this approach to the problem of population screening needs no further emphasis.

The use of a proximal subtotal gastrectomy with esophagoantrostomy produces achylia gastrica, as noted for the last 4 patients listed in table 8.

TABLE 8.—*Fecal excretion of radioactive vitamin B_{12} in various types of subtotal gastrectomy*

Patient	Age	Diagnosis	Type of gastrectomy	Degrees of free HCl before surgery	Microscopic appearance of resected stomach	Percent fecal excretion of vitamin B_{12} Co^{60}
ZR	59	Duodenal ulcer	Billroth I	—	Normal body mucosa	65
AT	53	Gastric ulcer	Billroth I	0	Normal body mucosa	63
JC	52	Duodenal ulcer	Billroth I	36	Normal body mucosa	25
GG	54	Gastric ulcer	Billroth I	11	Normal body mucosa	12
LK	68	Duodenal ulcer	Billroth II	48	Normal body mucosa	17
DP	69	Duodenal ulcer, gastric ulcer	Billroth II	46	Normal body mucosa	26
MO	63	Duodenal ulcer	Billroth II	76	Normal body mucosa	41
MJ	52	Gastric ulcer	Segmental	18	Gastric mucosal atrophy with intestinalization	86
BG	45	Gastric ulcer	Segmental	38	Normal body mucosa	42
AA	70	Duodenal ulcer	Segmental	133	Normal body mucosa	39
CS	57	Duodenal ulcer	Tubular	102	Normal body mucosa	18
LB	35	Duodenal ulcer	Tubular	63	Normal body mucosa	44
MS	43	Duodenal ulcer	Tubular	69	Normal body mucosa	43
MH	52	Cardiospasm	Proximal subtotal gastrectomy with esophagoantrostomy	35	Normal body mucosa	84
SG	65	Adenocarcinoma of stomach	"	0*	Normal body mucosa	74
LC	60	"	"	0*	Normal body mucosa	99
VN	35	Cardiospasm	"	†	Normal body mucosa	90

*Test performed immediately prior to operation revealed a normal excretion of vitamin B_{12} Co^{60} in the urine.
†This patient recently developed megaloblastic anemia 5 years after operation.

Patient SG, with adenocarcinoma of the stomach, had normal excretion of radioactive vitamin B_{12} in the urine immediately prior to the operation. There was no free acid on gastric analysis. The body mucosa was normal histologically, and the radioactive vitamin B_{12} studies indicated an achylia gastrica postoperatively. We may well ask: Does such an individual have an increased predilection for development of gastric cancer in the antral pouch because of the absence of intrinsic factor?

The body and fundus of the stomach are probably the sole sources of intrinsic factor. Studies of patients with all types of gastrectomy—Billroth I, Billroth II, segmental, tubular, proximal subtotal resections of fundus and body, and total gastrectomies—have confirmed this original opinion of Fox and Castle. Some authors have reported an increased number of cancers in the body of the stomach of patients with frank pernicious anemia, but our experience does not support this view. However, further evaluation of accumulated results from several clinics might shed more light upon this problem.

Summary and Conclusions

Although we cannot state categorically that atrophic gastritis and gastric cancer are causally related, current evidence suggests the likelihood of this relationship. A much higher correlation of severe gastric mucosal atrophy is found in persons with gastric cancer than in any other group except the pernicious-anemia patients. In the latter, the incidence of gastric cancer is 20 times that found in a comparable segment of the normal population. However, gastric carcinoma can exist in a stomach with all the histologic features of normalcy and a normal secretory capacity. We have never been aware of a demonstrable qualitative difference between carcinomas found under these varied circumstances.

The uniform absence in lower animals of gastric mucosal atrophy, megaloblastic anemia, adenomatous gastric polyps, and gastric cancer contrasts sharply with the situation in man; the well-known incidence of achlorhydria, gastric adenomas, mucosal atrophy, pernicious anemia, and cancer of the stomach suggests a definite etiologic relationship between gastric atrophy and gastric cancer.

At the University of Minnesota Hospitals, screening for achlorhydria and hypochlorhydria has proved an effective means of diagnosing a reasonable number of gastric cancers in the asymptomatic phase of the disease. During a 10½-year period, over 12,000 men and women have undergone a screening test to detect those persons likely to develop gastric cancer. The portion of the total population that must be examined periodically because of increased likelihood to develop gastric cancer has been reduced to 6½ percent. Economically, the problem of population screening becomes feasible through this method.

Adequate protection for the population at large appears to result from this approach. No case of gastric cancer in the University of Minnesota Cancer Detection Research Center would have been undetected if all other screening techniques except examination for achlorhydria and

hypochlorhydria had been omitted. The etiologic relationship of achlorhydria and atrophic gastritis with gastric cancer is strongly supported by these results.

In the achlorhydric-hypochlorhydric group the incidence of gastric cancer is 4.5 times greater, and in the pernicious-anemia group it is 21.9 times greater, than that in the same segment of the normal population. We believe our studies indicate practical and effective criteria for determining which segments of the total population should be screened, thus reducing to a practical size the group to be followed in the survey. Members of this group probably should be examined according to the following schedule:

(*a*) Pernicious-anemia patients—gastrointestinal roentgenograms at 6-month intervals.

(*b*) Achlorhydric-hypochlorhydric persons—gastrointestinal roentgenograms at 9-to-12-month intervals.

Additional studies of achlorhydric persons, by means of the Schilling test, indicate that about 5 percent have preclinical pernicious anemia. Further refinement of the groups that require repeated study may be possible through the application of tests of this kind.

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Incidence of Mammary and Pituitary Tumors in Hybrid Mice Treated with Stilbestrol for Varying Periods [1,2]

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In most experiments on the induction of mammary cancer by estrogen, male mice have been used because they do not get these tumors naturally. However, the incidence of cancer in females can be increased and the age at which it occurs, reduced. Burns and Schenken (*1*) made quantitative studies on the relationship between the administration of estrogen and mammary-gland carcinoma. From the time the mice were 2 weeks old, strain C3H males were injected weekly with estradiol benzoate in sesame oil, and this treatment was continued for varying periods. No tumors developed after 4 weeks of treatment but there was a low incidence after 8 and 12 weeks. The greatest effect was obtained after treatment for 16 weeks. A long interval of 6 to 16 months elapsed between cessation of estrogens and appearance of cancer. Suntzeff, Kirtz, Blumenthal, and Loeb (*2*) used nonbreeding females and male mice of strains D and A and injected them with estrogen for varied periods. They found that the incidence of tumors was zero, or very low, in animals injected for 3 to 4 months and reached a maximum in mice injected for 9 to 12 months. They also stated that the shorter the period of injections the longer the latent period.

Gardner (*3*) used F_1 hybrids of CBA and C57BL mice to determine the effect of estrogen on the incidence of mammary tumors. He described whole-mount preparations and sections of mammary glands of these estrogen-treated hybrids. The individual glands almost always showed a characteristic lack of uniformity in different areas whether or not the animals had mammary carcinoma. Nodules of denser epithelial tissue were present at random points. Some of the nodules were composed of groups of large alveoli, others of well-circumscribed clusters of small alveoli. Some nodules were so dense that their structure could not be determined from whole-mount preparations. Photographs of the glands showed concretion-like masses that were found in groups and chains, and the latter gave the ducts a beaded appearance. The intraductal masses

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were found in the glands of all estrogen-treated mice but rarely in untreated animals. Shimkin and Wyman (*4*) reported some of the advantages of administering estrogen in the form of solid pellets instead of repeated injections of the material dissolved in oily or aqueous vehicles. They showed the correlation between the concentration of the hormone in the estrogen-cholesterol pellet and the mammary-tumor response in C3H male mice. For example, they found that pellets containing 20 percent stilbestrol-in-cholesterol and weighing only 2 mg. produced tumors in 24 of 32 mice.

Richardson and Pearson (*5*) described the changes that occurred in the mammary glands during carcinogenesis of mammary tumors, in mice implanted with stilbestrol pellets. The mice used were F_1 hybrids of RIII $\times$ C57L. The mammary glands of animals sacrificed at 5 months of age showed large ducts lined with 1 layer of cuboidal epithelium. Within each duct, there was material that stained pink with hematoxylin and eosin and, in many instances, was organized into concretions. There was also a mild proliferation of acini. In the 6-month-old mice there were no essential differences to be observed. Hyperplastic nodules were seen in a number of instances, either in whole mounts or in sections. In the mice killed at 7 months of age there were striking differences. Many more hyperplastic nodules could be seen. Some of the medium-sized ducts were proliferative and lined with 2 or 3 layers of cells. Concretions in the ducts were very prominent. During the 8th and 9th months these changes were accentuated; there was an increase in the size and number of the hyperplastic nodules and a progressive dilatation of the ducts with multiple concretions. This was especially apparent in studying the whole mounts.

Materials and Methods

In this investigation 910 mice were used. There were 348 female and 381 male F_1 hybrids of a cross between RIII females and C57L males. In the reciprocal cross there were 91 females and 90 males. Mice of the RIII strain used in these experiments have a high incidence of mammary tumors in the breeding females and a low incidence in virgins. The C57L is a low-mammary-tumor strain.

Solid pellets of stilbestrol-cholesterol were prepared by a modification of the technique described by Shimkin and White (*6*) and were implanted subcutaneously by means of a 12-gauge trocar. Each pellet weighed 7 to 8 mg. and contained 20 percent stilbestrol and 80 percent cholesterol. Pellets were implanted into 8-week-old animals.

F_1 hybrids of RIII $\times$ C57L contain the mammary-tumor inciter as the female parent belongs to a high-mammary-tumor strain. The experiments in which these hybrids were used consisted of 3 major groups: 1) those receiving continuous estrogen stimulation after 8 weeks of age, 2) those receiving estrogen for a time limited by removal of the pellet after 4, 6, or 8 weeks, and 3) virgin females without treatment with stilbestrol. A relatively small number of mice were treated with stilbestrol for 4, 6, or 8 weeks and then injected intraperitoneally with

20 I.U. of prolactin (luteotrophin) 3 times a week until a total of 10 injections had been given. The purpose of using prolactin was to ascertain whether the incidence of mammary tumors would be increased or whether the tumors would develop sooner than in mice treated only with stilbestrol.

When mammary tumors were visible to the naked eye, the animals were killed by chloroform. A section of each tumor was saved for histologic diagnosis; usually the skins were removed, leaving the mammary glands adherent. Whole mounts were made of the third glands. These were stained with Meyer's hematoxylin and studied under the dissecting microscope. All mice that reached tumor age were checked once a week for tumors. Old mice without tumors were autopsied when death seemed imminent.

Observations

Mammary carcinoma.—Table 1 shows that when pellets of stilbestrol were implanted into (RIII × C57L)F_1 virgin females, at the age of 8 weeks, receiving continuous estrogen thereafter, a high percentage (86.8%) of the animals had mammary adenocarcinomas at an average age of 11.1 months. Although this hybrid animal tolerated treatment with stilbestrol better than many other strains, the average age of death for animals receiving stilbestrol for life was considerably lower than in the groups receiving estrogen for limited periods. When pellets were removed 8 weeks after implantation, the percentage of animals with mammary tumors (88.6%) was slightly larger than in females receiving continuous stimulation, but the average age when the tumors appeared was 16.9 months. When pellets were removed 4 weeks after they were implanted, there was a definite drop in the percentage of females developing mammary tumors (75.0%) and some increase in the average tumor age (19.6 months). In the control group of virgin females receiving no exogenous hormone stimulation, there was a marked decrease in the percentage of mice developing mammary tumors (62.5%), and the average tumor age (21.3 months) was slightly higher than in mice receiving stilbestrol for 4 weeks. Only a very small percentage (6.3%) of the untreated females had tumors before 16 months of age.

TABLE 1.—*Occurrence of mammary tumors in virgin females of (RIII × C57L)F_1 hybrids*

Duration of treatment (*weeks*)		No. of mice	Percent with tumors	Percent tumors before 16 months	Avg. age when tumor appeared (*months*)	Range in age (*months*)	Percent dead without tumor	Avg. age of death without tumor (*months*)
Estrogen	Prolactin							
For life	—	174	86.8	79.3	11.1	5.2–20.6	13.2	12.2
8	—	52	88.6	34.6	16.9	5.1–27.6	11.3	19.0
4	—	40	75.0	20.0	19.6	9.8–31.8	25.0	23.0
None	—	48	62.5	6.3	21.3	13.6–31.2	37.5	22.4
8	3.3	13	92.3	53.8	15.5	6.8–24.3	7.7	15.2
6	3.3	8	87.5	25.0	18.4	13.4–27.8	12.5	19.5
4	3.3	12	83.3	25.0	19.3	15.4–24.0	16.7	12.1

In the reciprocal cross, (C57L × RIII)F_1 hybrids, pellets of stilbestrol were implanted at 8 weeks of age. Because these pellets were not removed, the animals received continuous estrogen stimulation after implantation. The mice were examined regularly for pituitary tumors. In most instances, it was possible to tell whether or not a tumor was present by the shape of the animal's head. Mice were sacrificed when these tumors were apparent or when they became moribund. Some pituitary tumors were sectioned for microscopic examination. Two pituitary tumors were assayed for gonadotrophin content, according to the method of Ladman, Harris, and Runner (7), by measuring the ovulatory response in immature mice primed by prior injections of pregnant mare serum.

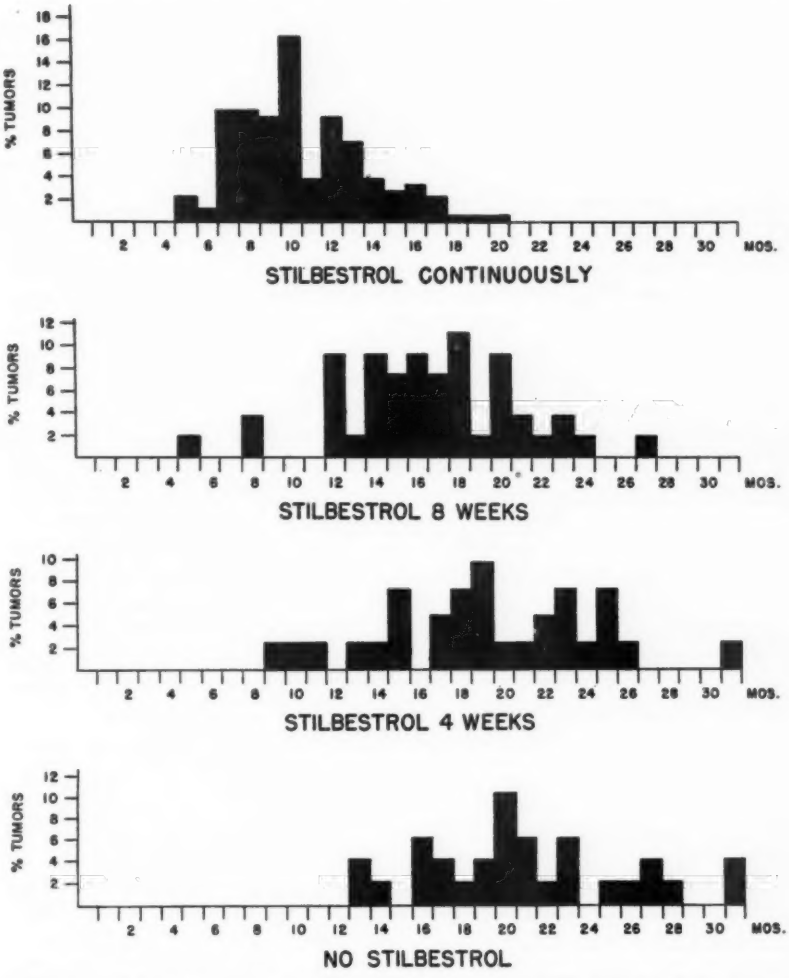
Table 1 gives data on the percentages of tumors and time of their appearance in mice injected with prolactin following treatment with estrogen. A small number of mice was used in this experiment so that the value of making comparisons with the previous group is somewhat questionable. The chi-square test shows that there is no significant difference in tumor incidence between the estrogen-prolactin-treated mice and those receiving stilbestrol only. Furthermore, prolactin does not decrease the average tumor age. The histogram (text-fig. 1) shows the percentages of tumors at the age, in months, when they were observed in females treated with estrogen, for varying periods, and in untreated females.

Table 2 shows that when pellets of stilbestrol are implanted into (RIII × C57L)F_1 normal males at 8 weeks of age and left for life, a high percentage (84.3%) of the animals have mammary adenocarcinomas at an average age of 10.9 months. These results are similar to those obtained by treating virgin females with stilbestrol. Only a relatively small percentage of the males had mammary tumors when the pellets were left in for 8, 6, or 4 weeks. The average tumor age of the males treated for 8 weeks (17.7 months) was about the same as that of the females treated for the same length of time (16.9 months). Eleven male mice were injected with prolactin following treatment with stilbestrol for 8 or 6 weeks; 1 mouse developed a tumor.

No mammary tumors appeared in 91 female and 90 male F_1 hybrids of the reciprocal cross (C57L × RIII) that received continuous estrogen stimulation after implantation of the pellet, at 8 weeks of age. Although

TABLE 2.—*Occurrence of mammary tumors in males of (RIII × C57L)F_1 hybrids*

Duration of treatment (*weeks*)		No. of mice	Percent with tumors	Percent tumors before 16 months	Avg. age when tumor appeared (*months*)	Range in age (*months*)	Percent dead without tumor	Avg. age of death without tumor (*months*)
Estrogen	Prolactin							
For life	—	230	84.3	80.9	10.9	5.4–25.1	15.7	13.3
8	—	34	8.8	0	17.7	16.5–19.0	91.2	26.5
6	—	56	10.7	3.6	18.7	10.4–24.6	89.3	23.4
4	—	48	2.1	2.1	11.2	(1 mouse)	97.9	26.6
8	3.3	5	20.0	20.0	8.0	(1 mouse)	80.0	21.1
6	3.3	6	0	0	0	—	100.0	25.4



TEXT-FIGURE 1.—Histogram showing percentage of mammary tumors at the age when they occur in (RIII × C57L)F_1 virgin female mice treated with stilbestrol, for varying periods, and in untreated females.

these mice are genetically the same as the other hybrids, the female parent does not have the mammary-tumor inciter.

Many whole-mount preparations of the third mammary glands of (RIII × C57L)F_1 hybrids were made. The glands of normal males in this cross have been described previously (*8*). Figure 1, the third gland of an untreated male, 2.1 months old, shows that the ducts are narrow and acini are absent. Figure 7, the third gland of a normal female 3.7 months of age, shows a uniform proliferation of small acini throughout the glandular area. Twenty males and 20 females, 3.7 months old, were killed after

treatment with stilbestrol for 8 weeks, and preparations were made of a third gland from each mouse. The male glands (fig. 2) closely resemble those of the females (fig. 8), except for the absence of the nipple and primary duct in the males. The ducts are usually much wider than in untreated mice, and there is a mild proliferation of acini. The material that fills the ducts also causes distention of many of the acini. Very small, hyperplastic nodules are present in 4 of the male glands and in 3 of the female glands. In the mammary glands of most of the tumor-bearing male and female mice that received a continuous supply of stilbestrol, the ducts are dilated and hyperplastic nodules are abundant, especially in the older mice (fig. 10). However, only small nodules are present in the glands of some of these mice (fig. 3). A material, which appears to be semisolid or exists in concretion-like masses (figs. 5, 6), is present in the ducts of the glands of the tumor-bearing mice that were treated with stilbestrol for life. The concretions vary greatly in size and number.

Ten male mice developed tumors following stilbestrol treatment for 4, 6, or 8 weeks. In the glands of 9 of these, many of the ducts are very slender and there is complete regression of acini except for those arranged in very small nodules (fig. 4). Some acini and larger nodules are present in the glands of the remaining mouse that developed a tumor. Mammary glands of tumor-bearing females that received stilbestrol for 4 or 8 weeks (fig. 12) often show many hyperplastic nodules but, in general, they are not so large or numerous as in old females that received treatment for life. The ducts vary greatly in width and seldom contain concretions. The mammary glands of untreated tumor-bearing females (fig. 11) are similar to those of mice that were treated for limited periods. The ducts are frequently wide, especially in the nipple region, in the glands of old virgin females. Acini are only moderately abundant.

Mammary glands of mice that were injected with prolactin following estrogen treatment were examined. A few female mice were sacrificed at the end of the prolactin injections. These glands show more acinar growth (fig. 9) than the glands of mice receiving estrogen alone. The glands of the tumor-bearing males and females of the estrogen-prolactin group closely resemble those of mice that received stilbestrol for 4 to 8 weeks. The gland of a tumor-bearing male of this group is shown in figures 13 and 14. Many of the ducts are very slender and acini are absent except for those composing very small nodules. The gland of a tumor-bearing female of the estrogen-prolactin group is shown in figure 15. Acini are only moderately abundant and the nodules are numerous but not large. The glands of a few females show more acinar proliferation than the one pictured.

Pituitary tumors.—A high percentage of the F_1 hybrids of C57L × RIII developed pituitary tumors. An assay of 2 pituitary tumors for gonadotrophin content indicated it was less, by a factor of 3, than in normal pituitaries. Sections of these tumors, stained with hematoxylin and eosin, were diagnosed as chromophobe adenomas. Table 3 shows that 67.0 percent of the females and 53.3 percent of the males had pitui-

TABLE 3.—*Occurrence of pituitary tumors in (C57L × RIII)F_1 hybrids implanted with stilbestrol pellets*

Sex	No. of mice	Percent with tumors	Average age when tumor appeared (*months*)	Range in age (*months*)	Percent dead without tumor	Average age of death without tumor (*months*)	Range in age (*months*)
♀	91	67.0	17.7	13.0–27.5	33.0	13.8	6.6–23.1
♂	90	53.3	16.3	11.5–24.1	46.7	15.0	9.4–27.0

tary tumors, but the average tumor age was less in the males. Table 4 shows the ages at which the pituitary tumors occurred. A few tumors were observed in females at 13 and 14 months of age. The highest percentages appeared at 15 and 16 months of age, and there was a gradual decrease in tumor incidence between the ages of 17 and 27 months. In the males, a few tumors appeared at 11, 12, and 13 months of age. There was a sharp increase in tumor incidence at 14 months of age. After the animals were 15 months old, the tumor incidence per month decreased. The average age of death in both females and males was less than the average tumor age. It is probable that if the animals had lived to be 16 months of age or older a much higher percentage of tumors would have been observed.

The mammary glands of 2 (C57L × RIII)F_1 hybrid mice that had pituitary tumors are illustrated. Figure 16, the mammary gland of a male 14.7 months old, shows an uneven distribution of acini. A small nodule composed of a cluster of large acini, which tend to lie in one plane, is present. The ducts are dilated and many concretions can be seen under higher magnification. There is no indication that the pituitary tumor stimulated proliferation of acini. Figure 18, the gland of a female 16.0 months old, also shows uneven distribution of acini and a small diffuse nodule. No large thickened nodules were found in any of the glands of the mice in this group, and many of the glands contained no nodules. Figure 17 is the mammary gland of a normal female, 8.3 months old, and shows a uniform distribution of small acini throughout the glandular area and no hyperplastic nodules.

A comparatively small number of pituitary tumors were observed in F_1 hybrids of RIII × C57L mice receiving continuous stilbestrol treatment. A high percentage of these animals with mammary tumors were sacrificed before the age when pituitary tumors were observed in the

TABLE 4.—*Percentages of pituitary tumors at different ages in (C57L × RIII)F_1 hybrids implanted with stilbestrol pellets*

Sex	Percentage of tumors at following age (*months*):									
	11	12	13	14	15	16	17	18	19	20–27
♀	—	—	4.4	2.2	15.4	12.1	8.8	7.7	2.2	14.3
♂	1.1	2.2	4.4	14.4	7.8	8.9	2.2	4.4	4.4	5.5

reciprocal cross. No pituitary tumors were found in animals receiving stilbestrol for 4 to 8 weeks or in the controls.

Other tumors.—One hundred male mice that did not develop mammary tumors as a result of estrogen treatment for 4, 6, or 8 weeks were autopsied at an average age of 27.0 months, when death seemed imminent. Two of these animals had adenocarcinoma of the kidney; 1 mouse, 27.0 months old, had received stilbestrol for 4 weeks; and the other, 29.1 months old, had received stilbestrol for 8 weeks. One kidney tumor was transplanted subcutaneously into 4 normal males but grew in only 1 mouse. It took 14 months for this tumor to become large enough to be transplanted again. One male mouse, 16.6 months old, had an osteogenic sarcoma of the right femur. This tumor was successfully transplanted. Two mice, one aged 25.7 months and the other 30.7 months, had squamous-cell carcinoma of the stomach. Lung nodules were present in 32 of these males. Histologic sections of lungs of 9 of these mice were made, and the nodules were identified as primary lung tumors.

Discussion

The results of injecting stilbestrol for varying periods of time show that the incidence of mammary tumors in virgin females of (RIII $\times$ C57L)F_1 hybrids was as high in mice receiving stilbestrol for 8 weeks as in mice receiving it continuously. The incidence of tumors was lower in mice receiving stilbestrol for 4 weeks, and still lower in the control group. The average tumor age was shortest in the group receiving treatment for life and became progressively longer as the period of treatment decreased; it was longest in the control group. Only a relatively small percentage of the males receiving estrogen for limited periods developed mammary tumors. These males were not castrated so that the androgenic hormones of the testes may have had some inhibiting effect on tumor formation. There is a long latent period between the discontinuance of treatment and the time when the tumor becomes visible to the naked eye. This ranged from 7.0 to 21.1 months in the males and from 1.1 to 28.8 months in the females.

A study of the mammary glands of the tumor-bearing males that had discontinued estrogen treatment is of interest. The 4 pairs of glands were observed under the dissecting microscope, and whole mounts were made of a third gland from each mouse. With the exception of 1 mouse the nodules were extremely small, the acini had completely regressed, and most of the ducts were slender. The virgin females in this hybrid have a fairly high mammary-tumor incidence. These tumors appear late in life and the average tumor age is 21.3 months. Work is in progress to find out whether or not a higher percentage of males will develop mammary tumors, after stilbestrol treatment for 4 to 8 weeks, when the experimental animals belong to a hybrid strain in which the virgin females develop mammary tumors early in life. A study of the mammary glands will also be undertaken.

Pullinger (*9*) applied large doses of estradiol cutaneously, at 20-day

intervals, to a high-cancer line of RIII mice. Two doses were given to ovariectomized females and 3 doses to normal males. Growth of the duct system and proliferation of acini occurred in response to the hormone. In the absence of further stimulation, the ducts became shrunken and the acini regressed and finally disappeared. Involution was complete in the spayed females some time between the 16th and 20th weeks of the experiment, after limited estrogen treatment, and in the males between the 24th and 28th weeks. In the majority of mammae, areas of nodular hyperplasia persisted on otherwise atrophic duct systems. Nine out of 40 males developed malignant tumors by this treatment in 5 months.

Daane and Lyons (*10*) injected estrone and pituitary mammotrophin into castrated C3H male mice. They found the combination slightly more efficacious than estrone alone in producing good duct growth and fair alveolar growth of the mammary glands, but after 45 days of treatment no tumors were detected over a period of a year. A small number of F_1 hybrids of RIII $\times$ C57L were used in this preliminary experiment to determine the effect of injecting prolactin after stilbestrol treatment. The results indicate that prolactin did not decrease the tumor age or increase the tumor incidence in either the females or the males. The mammary glands of female mice sacrificed at the end of the prolactin injections showed more acinar growth than the glands of mice receiving estrogen alone. In old, tumor-bearing mice the glands were similar to those of mice receiving estrogen for limited periods only. A few glands with hyperplastic nodules were sectioned, and the mammary tumors were diagnosed histologically. The hyperplastic nodules and tumors of the stilbestrol-prolactin group were similar to those of the estrogen group. These results indicate that the prolactin injections caused only a temporary stimulation of acinar proliferation, which was followed by a partial regression after discontinuance of treatment.

Gardner (*3*) observed that F_1 hybrids of C57BL and CBA had pituitary tumors after prolonged estrogen stimulation. The incidence of pituitary tumors was slightly lower in both male and female mice of CBA $\times$ C57BL than in the reciprocal cross. Both male and female mice of the C57BL strain transmitted this tendency to develop pituitary tumors to their first-generation hybrid young. The hypophyseal tumors appeared at relatively earlier ages in the males and the incidence of tumors was greater than in the females. In the F_1 hybrids of C57L $\times$ RIII used in this study, the pituitary tumors appeared earlier in the males, but the incidence of these tumors was less than in the females.

Kirkman and Bacon (*11*) reported renal tumors in hamsters that had been treated with estrogen. Tumors were found in the kidneys in nearly all of their male hamsters that had been treated with stilbestrol for 250 days or longer, but renal tumors did not occur in normal females in response to stilbestrol. Renal tumors are very rarely found in mice in this laboratory. The fact that 2 of 100 male mice, which had received stilbestrol for limited periods, developed kidney adenocarcinomas may indicate that the tumors were caused by the estrogen. It would be interesting to try

to induce the formation of kidney tumors in male mice by increasing the period of estrogen treatment in a strain of mice that would not die with mammary or pituitary tumors, at a relatively early age, due to the stilbestrol.

Summary

Male and female mice of (RIII × C57L)F_1 hybrids were implanted with stilbestrol pellets for varying periods. The mammary-tumor incidence was as high in females receiving treatment for 8 weeks as in those receiving it for life. Tumor incidence was lower in females receiving treatment for 4 weeks and still lower in the control group. The average tumor age depended upon the duration of treatment and was lowest in the group receiving treatment for life. It became progressively longer as the period of treatment decreased and was longest in the control group. The incidence of mammary tumors and the average tumor age in males, treated continuously with stilbestrol, were similar to that of the females. A comparatively small percentage of mammary tumors arose in males implanted with stilbestrol pellets for 4, 6, or 8 weeks.

No mammary tumors arose in (C57L × RIII)F_1 hybrids implanted with stilbestrol pellets, but a high percentage of the mice developed pituitary tumors. These tumors appeared earlier in the males, but the incidence was higher in the females.

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(*10*) Daane, T. A., and Lyons, W. R.: Effect of estrone, progesterone and pituitary mammotropin on the mammary glands of castrated C3H mice. Endocrinology 55: 191–199, 1954.

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PLATES

Vol. 18, No. 6, June 1957
423744—57——9

PLATE 70

All figures are photomicrographs of mammary glands of (RIII × C57L)F_1 hybrid male mice.

FIGURE 1.—Right third mammary gland of normal male. *Note* narrow ducts and absence of acini. Age 2.1 months. × 3

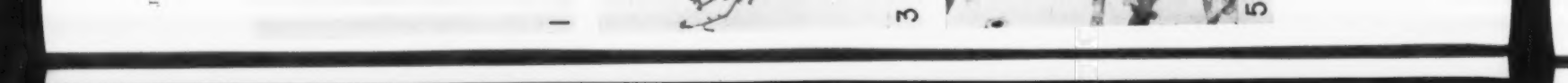
FIGURE 2.—Left third mammary gland of male sacrificed after treatment with stilbestrol for 8 weeks. The ducts have increased in width as well as in length and there is a mild proliferation of acini. Hyperplastic nodules are very small. Age 3.7 months. × 3

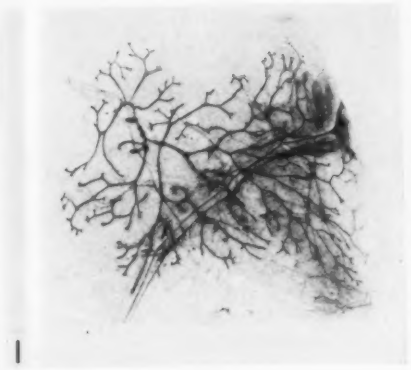
FIGURE 3.—Right third gland of tumor-bearing male receiving stilbestrol for life. Hyperplastic nodules are usually larger in old mice that have been stimulated continuously. Age 10.2 months. × 3

FIGURE 4.—Left third mammary gland of male treated with stilbestrol for 6 weeks. Mouse had a tumor of the third right mammary gland. *Note* the slender ducts and complete regression of acini except for those arranged in 2 small clusters. Larger nodules are seldom seen in old males of this group. Age 24.6 months. × 3

FIGURE 5.—Higher magnification of area in *lower right* of figure 3 to show concretions in the ducts. × 20

FIGURE 6.—Concretions in the duct of a mammary gland that was sectioned and stained to demonstrate alkaline-phosphatase activity. The concentric rings and radiating lines of the concretions can be seen in unstained whole mounts. Photographed under phase microscope. × 400

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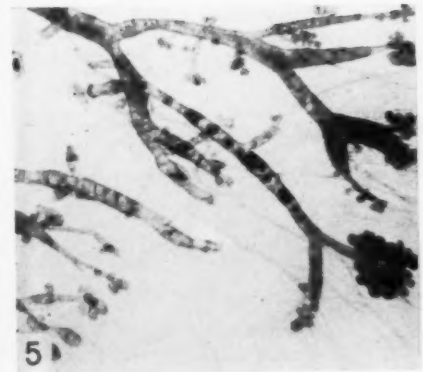
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Richardson

423744—57——9

PLATE 71

All figures are photomicrographs of whole-mount preparations of mammary glands of (RIII × C57L)F_1 hybrid female mice.

FIGURE 7.—Left third gland of normal female. There is a uniform proliferation of small acini. Age 3.7 months. × 3

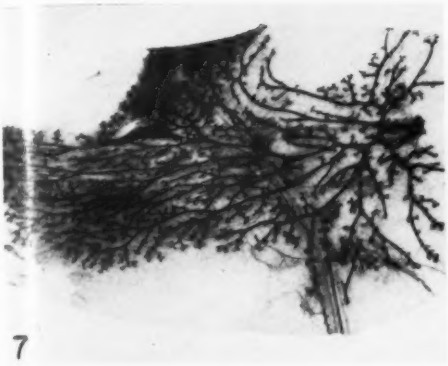
FIGURE 8.—Left third gland of female sacrificed after treatment with stilbestrol for 8 weeks. *Note* the distention of acini in the lower part of the gland and paucity of acini in other areas. Age 3.7 months. × 3

FIGURE 9.—Left third mammary gland of female injected with prolactin following stilbestrol treatment for 4 weeks. The gland shows more acinar growth than those of females receiving estrogen alone. Age 5 months. × 3

FIGURE 10.—Left third mammary gland of tumor-bearing female treated with stilbestrol for life. This shows the wide ducts and abundance of nodules usually found in mice at this age. Age 10.0 months. × 3

FIGURE 11.—Left third gland of untreated virgin female showing wide ducts frequently found in old animals. Mouse had a tumor of first right mammary gland. Age 20.7 months. × 3

FIGURE 12.—Left third mammary gland of tumor-bearing virgin female treated with stilbestrol for 4 weeks. Nodules were not as large or as numerous in this group as they were in many of the old mice which had been estrogen-treated for life. Age 19.8 months. × 3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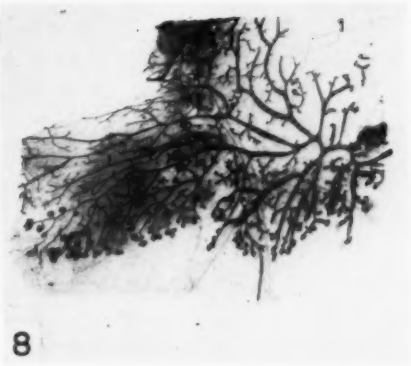
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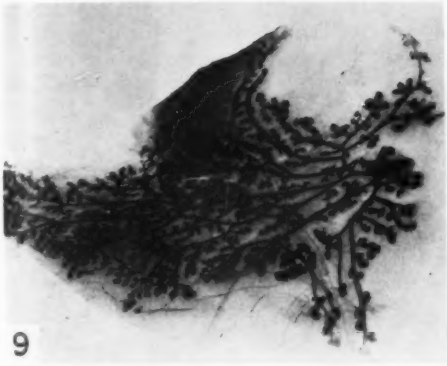
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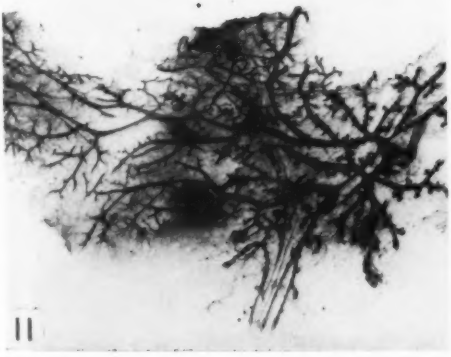
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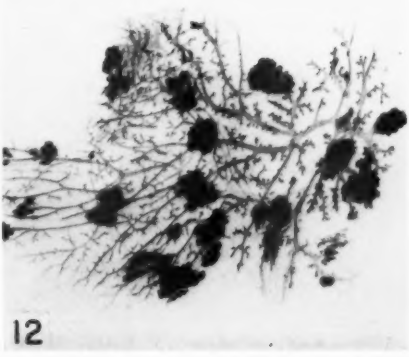
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PLATE 72

All figures are photomicrographs of whole-mount preparations of mammary glands.

FIGURE 13.—Left third gland of tumor-bearing male of the RIII × C57L cross treated with estrogen and prolactin. This gland resembles those of males receiving limited estrogen treatment only (fig. 4). Very small nodules are present. Age 8.0 months. × 3

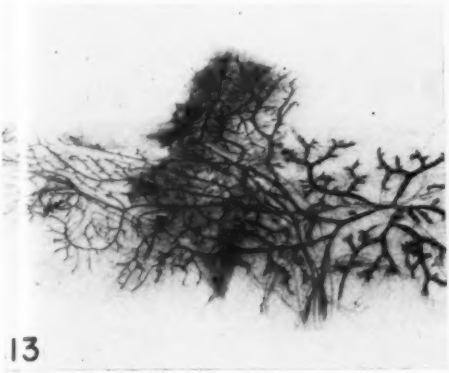
FIGURE 14.—Higher magnification of a nodule shown in figure 13. This shows a cluster of acini, diffusely arranged. × 20

FIGURE 15.—Left third gland of a tumor-bearing female of the RIII × C57L cross treated with estrogen and prolactin. This gland resembles those of females receiving estrogen only for a limited time (fig. 12). Age 16.0 months. × 3

FIGURE 16.—Left third gland of an estrogen-treated male of the C57L × RIII cross. *Note* uneven proliferation of acini and 1 diffuse nodule of large acini. This mouse had a pituitary tumor. Age 14.7 months. × 3

FIGURE 17.—Right third gland of an untreated virgin female of the RIII × C57L cross. There is a uniform distribution of small acini and no hyperplastic nodules. Age 8.3 months. × 3

FIGURE 18.—Left third gland of an estrogen-treated female of the C57L × RIII cross. *Note* uneven distribution of acini and a small flat nodule. This mouse had a pituitary tumor. Age 16.0 months. × 3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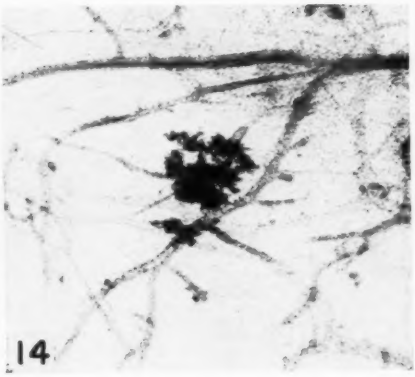
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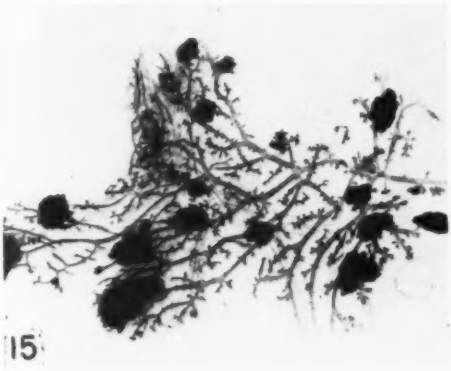
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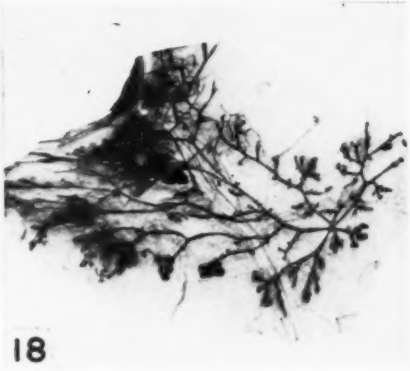
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423744—57——10

Virus of Avian Erythroblastosis. IV. *p*H and Thermal Stability [1,2]

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In the course of studies on the virus of avian erythroblastosis (*1–5*), it was desirable to determine the stability of the infectious agent under various laboratory conditions. An investigation has been made of the effects of hydrogen-ion concentration and of temperature and storage on the infectivity of the agent and on the morphology of the characteristic plasma particles associated with the disease. A description of the experiments and a comparison of the properties of the erythroblastosis virus with those of the agent of myeloblastosis (*6*, *7*) are the subject of this report.

Materials and Methods

In these experiments the Engelbreth-Holm strain "R" virus was used (*8*), as in the previous work on avian erythroblastosis in this laboratory (*1–5*). The results of early passages of the apparently pure strain of the disease in this country have been described (*2*). No changes have been observed thus far either in host response (*3*, *4*) to the agent or in the pathologic manifestations of the leukemia.

Plasma obtained from diseased birds near death (*2*, *3*) contained the virus for the present studies. Blood was drawn into heparin by heart puncture; the cells were removed by centrifugation; and the plasma was filtered first with celite #512, then through a Selas filter of 02 porosity, all by the usual procedures (*2*). Donor birds were inbred White Leghorn chickens of line 15 (*9*) (Regional Poultry Research Laboratory, East Lansing, Mich.) routinely employed in this laboratory for studies on both myeloblastosis and erythroblastosis.

Bioassay of virus infectivity of the various test mixtures was made by analyses based on the length of the period, in days, between inoculation of the virus and the *death* of the host with the procedures established for the erythroblastosis virus (*2*). The intravenous inoculum was 0.1 ml. of the test fluid for each chick of 30 birds per dose group. Examination of the specific particles in the electron microscope was made after sedimen-

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[2] This work was supported by a research grant to Duke University from the American Cancer Society, Inc., on recommendation of the Committee on Growth; by a grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; and by the Dorothy Beard Research Fund.

tation onto an agar surface as already described (*1*, *2*). The preparations for this procedure were diluted with 0.14 *M* NaCl in 0.001 *M* sodium phosphate solution of *p*H 7.0.

For the *p*H stability studies, a pool of plasma was collected from 4 donor birds. A series of preparations was set up with 2.5 ml. of the pooled plasma mixed with buffer solution containing sodium acetate, sodium phosphate, and histidine hydrochloride, with each salt in 0.01 *M* final concentration; HCl or NaOH was added to give the desired *p*H values and sufficient calcium-free Ringer's solution to yield a final volume of 10 ml. The mixtures were kept at 2° C. After 1 hour, 24 hours, and 72 hours, 3 ml. samples of each mixture were withdrawn, neutralized, and then made up with calcium-free Ringer's solution to make a total plasma dilution of 10-fold. Control samples of the initial plasma pool had been diluted fourfold with calcium-free Ringer's solution and stored at −78° C. (under crushed CO_2 ice). For comparison with the test samples, 1 control sample was thawed when the samples of test mixture were neutralized and diluted with calcium-free Ringer's solution to a final 10-fold plasma dilution. *p*H measurements were made with the glass electrode at 23 to 25° C.

For the thermal-stability experiments at 56°, 51°, 46°, 37°, 25°, 2°, and −22° C., plasmas from 4 diseased birds were pooled. Crystalline penicillin G (500 units per ml.) and streptomycin sulfate (0.1 mg. per ml.) were added. The plasma pool, undiluted, was divided into 1 ml. lots, which were stored in 13 × 100 mm. tubes closed with rubber stoppers. All samples, except those to be kept at 2° C., were frozen in an ethanol-solid CO_2 mixture, and those to be stored at −22° C. were transferred immediately to a chest at that temperature. Samples to be used in experiments at temperatures higher than 2° C. were kept at −78° C. until needed. These were thawed in water at room temperature and placed immediately in an ice bath. For the individual experiments, the tubes were immersed in a water bath at the selected temperature (controlled to ±0.1° C.) to a depth of about two thirds the tube length. At the end of the heating period, the tubes were returned to the ice bath and portions of the contents were diluted for inoculation and electron micrography. In the experiments at −22° and −78° C., the tubes were thawed at room temperature in a water bath and chilled immediately in ice, and portions of the preparation were diluted as above.

In each heating experiment the zero-time sample was simply thawed and treated as described for the −78° C. control samples. In the experiments at temperatures of 25° C. and higher, all samples in each experiment were titrated in the same weekly hatch of chicks, 14 to 16 days of age. The longer-term experiments at 2° C. and lower temperatures were made with chicks of the same age from different hatches, but all were from the same laying flock.

Results

*p*H *stability*.—As seen in table 1, the *p*H range included 5 levels between *p*H 3.5 and 9.6. There was a mishap with the mixture that was to be at

*p*H 8.7, which was corrected immediately after the first hour. After each interval each of the mixtures was inoculated (0.1 ml. inoculum per test chick) at the 10-fold plasma dilution described above and at a further 10-fold dilution made with BSA-Simms' solution (*2*). Thus the volumes of plasma given each chick were 10^{-2} and 10^{-3} ml., respectively. The control for each interval was inoculated at the 10-fold dilution level and at 2 further dilutions, so that the plasma volumes for the respective dose groups were 10^{-2}, 10^{-3}, and 10^{-4} ml. per chick. Thirty-nine dose groups (1,170 chicks) were required for the whole experiment and, consequently, not enough birds of the same age were available over the 4-day period. For this reason it was necessary to use dose groups consisting of hosts of varied age. On the day of the first set of inoculations, each group comprised ten 10-day-old chicks, eleven 26 days old, and nine 33 days old. Previous experience (*3*) has shown that these differences in age would not be expected to contribute greatly to individual variation in susceptibility to erythroblastosis.

TABLE 1.—*pH stability of the infectivity of the virus of avian erythroblastosis at 2° C.*

Incubation time (*hours*)	*p*H of incubation mixture	Median log latent period*	Log percent residual infectivity	Percent residual infectivity
1	3.5	1.023	0.94	9
	5.1	0.971	1.91	81
	7.0	0.968	1.96	98
	7.0	0.960	2.11	129
	9.6	0.964	2.06	115
	Control	0.966	2.00	100
24	3.5	>1.2	<2.00	<0.01
	5.1	0.978	1.76	58
	7.0	0.946	2.44	275
	8.7	0.994	1.56	36
	9.6	1.008	1.31	20
	Control	0.970	2.00	100
72	3.5	>1.2	<2.00	<0.01
	5.1	0.989	1.52	33
	7.0	0.978	1.74	60
	8.7	1.011	1.12	13
	9.6	1.056	0.30	2
	Control	0.964	2.00	100

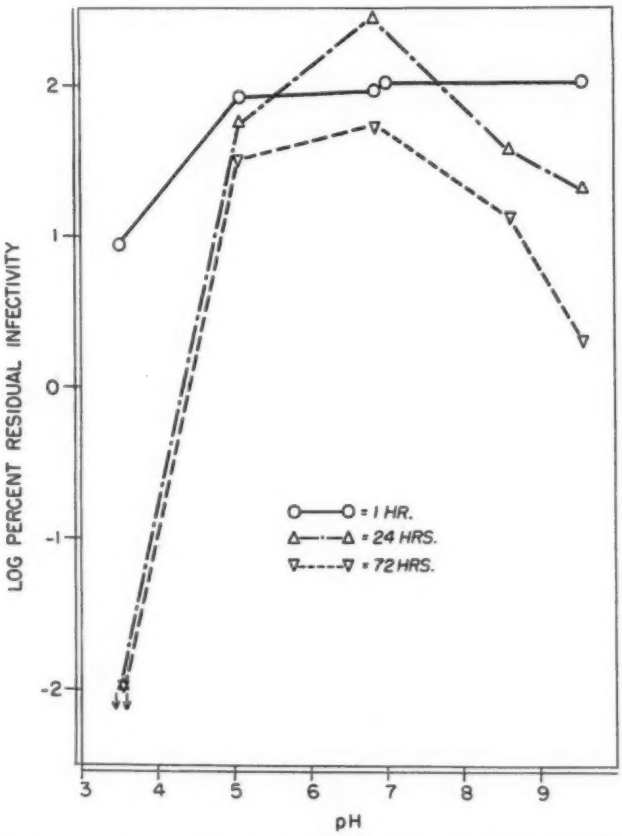
*Time from inoculation to death of the bird.

The essential data collected in these experiments are given in table 1 and summarized graphically in text-figure 1. In text-figure 1, the levels of infectivity of test samples [see (*2*) for statistics used in these calculations] and comparisons with corresponding controls are shown for each time period. The median log latent period values[3] for the control samples (stored at −78° C.) were 0.966, 0.970, and 0.964 log days after

[3] All median log latent-period values employed in both *p*H and thermal-stability studies and used in calculations of residual infectivity were computed at the level of response to 10^{-2} ml. of control plasma by means of the linear regression of standard slope 0.054 (*3*).

1, 24, and 72 hours, respectively. The *p*H of the control plasma pool was 7.7.

The property of infectivity of the virus was stable for 1 hour between *p*H 5 and *p*H 9.6 but decreased markedly at *p*H 3.5. After 24 hours, the infectivity had decreased at the *p*H values 5 and 8.7 and somewhat more



TEXT-FIGURE 1.—Effect of hydrogen-ion concentration on the infectivity of the virus of erythroblastosis during intervals of exposure of 1, 24, and 72 hours at 2° C. The *arrows* downward indicate values of less than 0.01 percent residual infectivity.

at 9.6, and at *p*H 3.5 it dropped below the limit of sensitivity of the assay procedure. The pattern after 72 hours was similar, with a further decrease in activity at each *p*H value above 3.5.

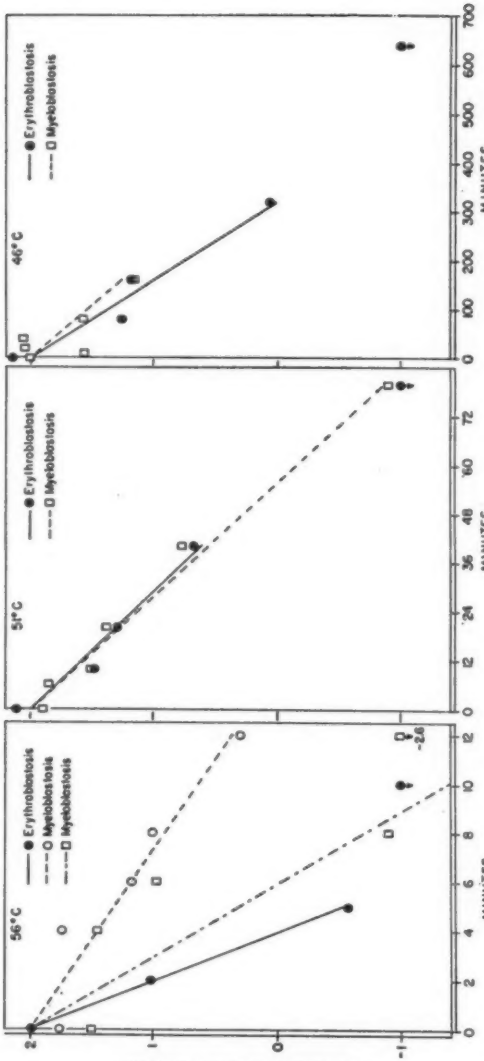
It must be noted that the sequence of experiments with erythroblastosis differed from that with myeloblastosis. In the present instance, the same mixtures were used for the different time periods at the respective *p*H levels; text-figure 1 shows not only the relative influence of various hydrogen-ion concentrations but also the absolute effects of *p*H

and time of storage at all levels. This was possible because of the availability of a sufficiently large group of birds and access to an essentially constant standard. With the virus of myeloblastosis, however [text-fig. 1A in (*6*)], there was no constant control, and only 3-day-old birds were suitable for titration. Thus, it was possible in that case to determine only the relative effects of *p*H under the conditions of the individual intervals of storage. There was no change in the appearance of the specific erythroblastosis plasma particles in electron micrographs nor was there any significant change in their concentration.

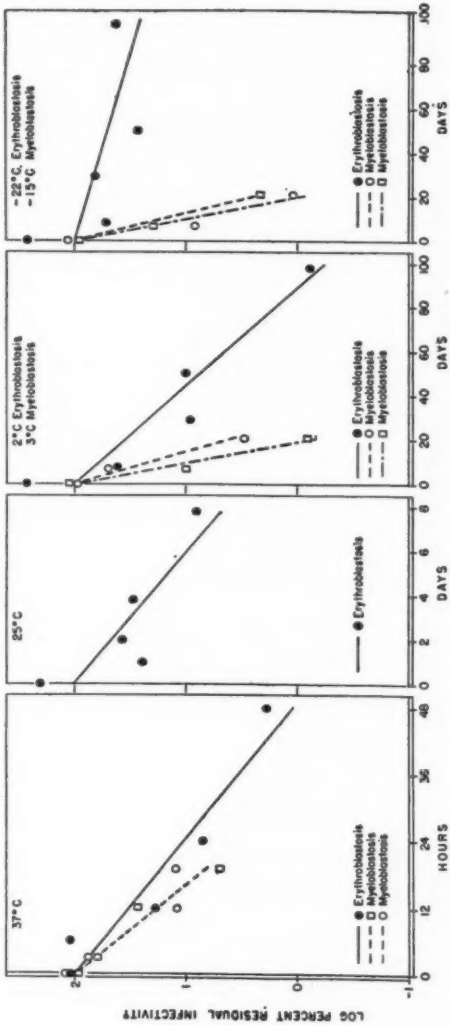
Thermal stability.—The data from the studies on the influence of temperature on virus infectivity are summarized in text-figures 2 and 3. It will be recalled that the plasma containing the virus was heated or incubated undiluted in the various experiments. When the study at each temperature was complete, 3, 2, or 1 10-fold dilutions were made of each control and test sample, the number of dilutions depending on the level of significant response expected. Calculations were made of the median log latent period (at the level of response to 10^{-2} ml. plasma, as in the case of the *p*H stability studies) for each sample titrated, including the zero-time control. Inspection of the plots of median log latent period against time of heating suggested that the relationship was essentially linear, which also appeared to be the case with the virus of myeloblastosis (*7*). Accordingly, the results were plotted and straight lines were constructed by the method of least squares, and the median log latent period at the zero-time intercept was taken as the index of initial activity. On the basis of this value, the median log latent periods of the treated samples were then transformed to percentage of residual infectivity and plotted as shown in text-figures 2 and 3.

In the experiments at 2° and −22° C., which extended over many weeks, the infectivity of the plasma stored at −78° C. was assumed to be constant, as it actually seemed to be during the course of the experiments, and the variation in response to it from one weekly hatch of chickens to another was assumed to be due to host variation. The responses of the chickens to test materials stored at 2° C. or −22° C. were therefore compared, at each time interval, with the responses of chickens from the same hatch to plasma stored at −78° C. With the responses to this preparation as the standard, the relative potency ratios of the experimental preparations were computed in terms of residual infectivity and plotted as seen in text-figure 3. An additional measurement was made, at 209 days, of the infectivity of virus stored at −22° C. The result confirmed the slope indicated in the corresponding graph in text-figure 3.

The apparently linear decrease in activity with time, expressed as log percent of the control value, shown in text-figures 2 and 3, is characteristic of a first-order reaction. Multiplying by 2.3 the values of the slopes of the lines in the respective graphs results in the values of the reaction constant, k (*10*), which are listed in table 2 and compared with analogous findings for the virus of myeloblastosis (*7*). In text-figure 4, the reaction rates found with the 2 viruses are plotted against the reciprocals of the



TEXT-FIGURE 2.—Thermal stability, at 56°, 51°, and 46° C., of the infectivity (*continuous line*) of the virus of erythroblastosis in filtered plasma. The *broken lines* show, for comparison, analogous data obtained in previous work (*7*) with the virus of myeloblastosis. The *arrows* downward indicate values of less than 0.1 percent residual infectivity.



TEXT-FIGURE 3.—Thermal stability, at 37°, 25°, 2°, and −22° C., of the infectivity (*continuous line*) of the virus of erythroblastosis in filtered plasma. The *broken lines* show, for comparison, analogous data obtained in previous work (7) with the virus of myeloblastosis.

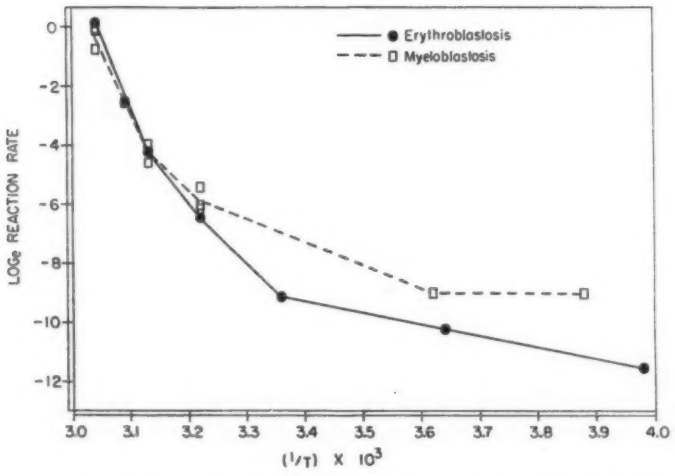
absolute temperatures of the experiments. It would appear that the relations observed with the respective agents were essentially linear in the range of 56° to 46° C. but showed pronounced deviation below this level. It is of interest that the rates of inactivation of the viruses were very similar at the higher temperatures. At temperatures below 46° C., however, it was clear that the agent of erythroblastosis was consistently more stable than that of myeloblastosis.

There was no evidence of significant change in the number or morphology of the virus particles under the conditions of the experiments.

Table 2.—*Reaction constants of inactivation of infectivity at various temperatures for the viruses of avian erythroblastosis and myeloblastosis*

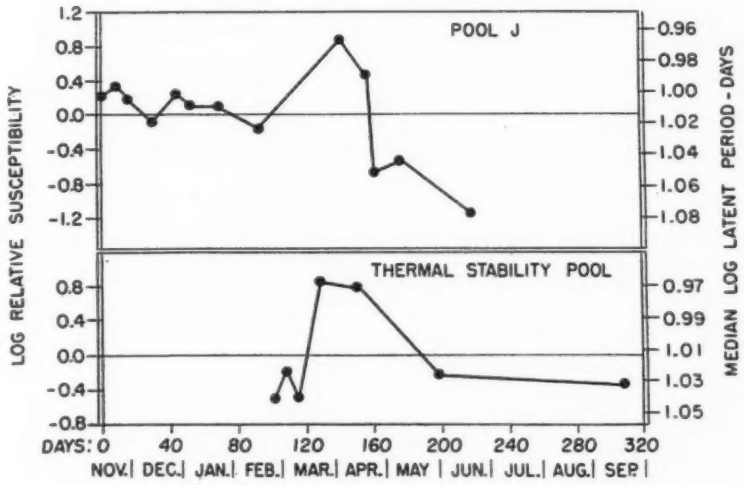
Temperature (° C.)	Reaction constant k (*min.* $^{-1}$)	
	Erythroblastosis	Myeloblastosis*
−78	Essentially 0	
−22	7.8×10^{-6}	
−15		1.2×10^{-4}
2	3.6×10^{-5}	
3		1.2×10^{-4}
25	2.5×10^{-4}	
37	1.6×10^{-3}	2.8×10^{-3}
46	1.4×10^{-3}	1.4×10^{-2}
51	8.0×10^{-2}	7.8×10^{-2}
56	1.17	0.78

*From reference (7).



TEXT-FIGURE 4.—Rates of inactivation of the infectivity (*continuous line*) of the virus of erythroblastosis at various temperatures. The *broken line* shows, for comparison, analogous data obtained in previous work (7) with the virus of myeloblastosis.

Text-figure 5 shows the results of repeated titrations, in different hatches of 14- to 16-day-old chicks, of 2 pools of virus in plasma covered with crushed CO_2 ice and stored at −78° C. Pool J was employed in the immunologic studies already described (*4*); the material for the thermal-stability pool has been described in the methods section of this paper. The data are plotted in terms of relative host susceptibility about the level of the mean log latent periods observed with the preparations at the respective time intervals.



TEXT-FIGURE 5.—Mean chicken response to 2 different plasma pools containing the virus of erythroblastosis at intervals during storage of filtered plasma at —78° C. under crushed CO_2 ice. Pool J provided the virus for the immunologic studies described in another report (*4*), and the second pool (the lower chart) was used for the thermal-stability experiments reported herein (text-figs. 2–4).

The findings summarized in text-figure 5 reveal considerable variation in infectivity of the virus or, conversely, in the resistance or susceptibility of different hatches of the test hosts at different times. That much or all of the variation was related to differences in response of the test groups is suggested by the striking parallel in the results with the 2 separate preparations stored under like conditions. It is of much interest that the period of relatively high host susceptibility, in March and April 1956, coincided with an outbreak of infectious bronchitis in the young chicks in the brooder room. With the elimination of bronchitis by isolation measures, resistance of the hosts returned approximately to the initial level. The results indicate that Pool J remained at about the same level of activity for 5 months, while the thermal-stability pool did not change appreciably in more than 7 months. These findings on the stability of erythroblastosis virus at −78° C. are similar in principle to those observed by Bryan (*11*) in the study of the Rous sarcoma virus.

Discussion

The results obtained in the present experiments reveal quite clearly the properties of the virus of avian erythroblastosis in relation to the influence of hydrogen-ion concentration and temperature of heating and storage on the stability of the infectious capacity of the agent. As such, the findings broaden knowledge about an agent responsible for direct induction of malignancy and are of much practical value in the prediction of its behavior under conditions like those employed in these studies. Of greater significance for the total problem of avian leukosis, however, is the additional basis for comparison of the virus of erythroblastosis with the agent of the sister leukemia, myeloblastosis.

Erythroblastosis and myeloblastosis are distinct diseases; the former involves cells of the erythroid series (*8*, *12*), and the latter presumably affects myeloblasts or progenitors of the myeloblasts (*13*, *14*). Despite pronounced differences in the cells specifically parasitized and in the well-recognized pathologic characteristics of the diseases, questions have been raised (*8*) on the interrelationships with respect to the etiologic agent or agents of the 2 conditions. This problem of the avian leukemias is but one aspect of analogous questions (*15*) concerned with all forms of avian leukosis. Systematic studies in this laboratory, however, have provided direct evidence that the diseases are caused by distinct though closely related entities. The distinction between the agents has been evident from the difference in (*a*) the respective patterns of host response (*2*); (*b*) the order of magnitude of "virulence" of or host susceptibility to the agents (*2*); (*c*) host susceptibility with respect to age; (*d*) activity to dephosphorylate adenosinetriphosphate (*2*, *16*); (*e*) immunologic identity (*4*). That the agents are closely related, however, has been shown (*4*) by the results of neutralization with homologous and heterologous immune serums and cross-precipitation experiments with specific chicken immune serums.

The findings in the present work have revealed further similarities and differences. Within the limits of the conditions imposed and the methods of study, the *p*H region of optimum stability and the range of relative stability of the virus of erythroblastosis did not differ qualitatively from those of the agent of myeloblastosis. In thermal stability, both agents behaved alike at the higher temperatures. However, in the lower range, about 37° C. and below, there was a difference which, on the basis of both magnitude and consistency, may be regarded as definitely significant. This difference constitutes another criterion that distinguishes the agents as separate biologic entities.

A long-standing difficulty in the study of the virus of erythroblastosis (as well as that of myeloblastosis) is the instability of the agent under ordinary laboratory conditions of storage. As illustrated by the present findings, much of this can be eliminated by storage at −78° C., under which conditions infectivity was maintained at a high level for at least several months. The agent of another avian malignancy, the Rous sarcoma, is highly stable (*11*) for long periods at this temperature. Re-

peated studies in this laboratory have indicated that the virus of myeloblastosis also maintains constant or slowly diminishing activity for several months under the same conditions. Access to the avian leukemia viruses, for use as standard preparations or for serologic studies that depend on known activity, aids greatly in the investigations of agents whose titration is both time-consuming and expensive.

Summary

Studies have been made on the *p*H and thermal stability of the virus of avian erythroblastosis. At 2° C. the *p*H range optimum for stability is in the region of *p*H 5 to 8.7, with the maximum at about *p*H 7. This was comparable with results observed with the agent of avian myeloblastosis.

Thermal stability was investigated at 56°, 51°, 46°, 37°, 25°, 2°, −22°, and −78° C. At each temperature, inactivation progressed as a reaction of the first order. As in the case of the virus of myeloblastosis, the rates of inactivation, expressed as logarithms, were essentially linear with the reciprocals of the absolute temperatures between 46° and 56° C. Below 46° C., the curve deviated sharply. Comparison of the relationships found with the 2 viruses showed that the agent of erythroblastosis was significantly more stable at the lower temperatures than that of myeloblastosis. Infectivity of the virus of erythroblastosis remained essentially constant for several months at −78° C.

There was no alteration in the number or electron microscopic characteristics of the virus particles under the conditions of the studies.

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Transplantable Sarcomas Induced in Rats by *N,N*-Dimethyl-*p*-(1-naphthylazo)aniline[1]

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The carcinogenic potentialities of compounds similar in chemical structure to *N,N*-dimethyl-*p*-phenylazoaniline (*p*-dimethylaminoazobenzene) are under study in this laboratory. Induction of malignant tumors of the liver in Osborne-Mendel rats by feeding in the diet either *N,N*-dimethyl-*p*-(1-naphthylazo)aniline [*p*-dimethylaminobenzene-1-azo-1-naphthalene (DAN)] (*1*) or *N,N*-dimethyl-*p*-(2-naphthylazo)aniline (*p*-dimethylaminobenzene-1-azo-2-naphthalene) (*2*) and induction of hemangioendothelioma in C57BR mice by painting with *N,N*-dimethyl-*p*-(2-naphthylazo)aniline (*3*) have been reported. This paper deals with the induction of sarcomas in Osborne-Mendel rats by subcutaneous injection of *N,N*-dimethyl-*p*-(1-naphthylazo)aniline; it includes a report on the successful transplantation of these tumors and a description of their morphology in the generation in which they arose and in subsequent transplant generations.

Material and Methods

Twenty Osborne-Mendel rats, 10 males and 10 females about 3 months old, were housed 5 to a cage and fed Purina chow pellets. Each rat received a subcutaneous injection of 0.5 ml. of a 2.5 percent solution of *N,N*-dimethyl-*p*-(1-naphthylazo)aniline in corn oil once a week for 1 year. No controls injected with corn oil alone were carried through at the time of these experiments. All animals were examined for tumors at regular intervals and the experiment was terminated at the end of 20 months. Animals that developed tumors were killed when their general health began to decline. Other rats were killed if they appeared sick and the rest at the termination of the experiment. Each primary tumor was transplanted subcutaneously in several male Osborne-Mendel rats. The rats with tumor transplants were examined at regular intervals to note the growth of the transplants and the general health of the animals. Rats were killed while still in good health, when the tumors were ready for transplantation. Six transplant generations are described in this report.

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

All animals, in both the primary-induction experiment and the transplantation series, were autopsied and tissues were taken for microscopic examination. Tissues were fixed in Zenker's acetic fluid and microscopic sections were stained routinely with hematoxylin and eosin.

Results

Two weeks after the first injection of DAN solution, small nodules, about 3 to 5 mm. in diameter, were felt in 5 rats, all females, at the site of injection. After 10 weeks, a pea-size nodule was found in another female rat at the site of injection. No further injections were given to these rats after the nodules were observed. No tumors were ever palpated in the other rats. The first 5 tumors, which appeared after 2 injections of DAN, increased in size rapidly; in 6 to 8 weeks they measured about $6 \times 4 \times 3$ cm. and weighed 30 to 130 gm. These primary tumors were large subcutaneous growths, with a diameter range of 3 to 7 cm. and mean weight of 55 gm. In 1 rat the tumor consisted of a single nodule weighing 130 gm. and measuring 7 cm. in diameter. In the other 4 rats, growths consisted of 2 separate nodules weighing 30 to 90 gm. and measuring 4 to 7 cm. in diameter. The majority of the tumors were adherent to the dermis and underlying axillary tissue, but no extensions into the adjacent tissue were visible. These tumors were usually firm, smooth in texture, and easily cut. As a general rule, the cut surface of the tumor (fig. 1) was pearly gray. However, larger tumors usually had either soft, spongy areas, yellowish-brown in color, or cystic areas with clear, yellowish, viscous fluid in them.

Of the 3 primary tumors that we attempted to transplant, all grew and were carried through several generations. They grew in 100 percent of animals and in 60 to 90 days attained a size of 6 to 10 cm. in diameter and a weight of 80 to 120 gm. The macroscopic features of transplanted tumors closely resembled those of the primary tumors. The first metastases to the lungs were found in the third and subsequent transplant generations.

The sixth tumor, which appeared after 10 injections of DAN, grew slowly when compared with the first 5. It attained a diameter of only 2 cm. and weighed only 6 gm. 6 weeks later.

Histopathology of Tumors

Sarcomas.—The tumors induced in the first 5 rats, all the transplant generations grown from them, and the mestastases to the lungs were similar in histology. They were sarcomas composed chiefly of large oval, elongated, or spindle cells that frequently showed moderate nuclear and cellular pleomorphism but no bizarre formations. Many of the nuclei were pale-staining but had a condensation of chromatin at the nuclear membrane and prominent nucleoli (fig. 2). Other nuclei were hyperchromatic, with coarsely clumped chromatin material throughout. The nucleus occupied a large portion of the cell and frequently caused bulging

of the cell wall. Mitotic figures were frequent and often atypical. Longitudinal fibrils were visible within the pale eosinophilic cytoplasm, and, when sectioned in the longitudinal plane, cytoplasm streamed out in a long tapering trail that terminated in a filamentous process. Cell borders were not distinct and usually the cytoplasm merged imperceptibly into fine pericellular collagen. The central portion of the tumors was often edematous and the cellular elements were rather widely separated (fig. 2). Liquefaction necrosis of the central portion of the tumor was apparent in some instances. Around the margins and adjacent to blood vessels, the tumor was more cellular and the cells appeared to be better preserved (fig. 3). Intersecting bundles of fibrous tissue gave a typical herringbone pattern to some areas of tumor (fig. 4). In several of the tumors (fig. 5), the striated muscle was invaded but not the overlying dermis and epidermis. The general microscopic features of the transplanted tumors (fig. 6) and lung metastasis (fig. 7) resembled the primary tumor but the cells were more pleomorphic and hyperchromatic.

Carcinoma.—The sixth primary tumor was a carcinoma. It was composed of proliferating and often distended ducts lined by atypical cuboidal epithelial cells, which were sometimes arranged in multiple layers. The chromatin of the large, rounded nuclei was concentrated chiefly at the nuclear membrane and in 1 or more prominent nucleoli. Nuclear polarity was poorly maintained and mitotic figures were moderately frequent. The ducts were irregular and poorly formed, and cords of epithelial cells invaded the surrounding stroma (fig. 8). A few mammary ducts present in subcutaneous tissue adjacent to the tumor suggest the probable origin of the carcinoma.

Discussion

Transplantable subcutaneous fibrosarcomas of rats are common. Dunham and Stewart (*4*), in a survey of transplantable and transmissible animal tumors, listed 9 induced or spontaneous tumors in rats. However, most of the tumors were induced after exposure to aromatic hydrocarbons. Yoshida (*5*), in 1934, reported induction of sarcomas and hepatomas in rats after prolonged subcutaneous injections of 4-*o*-tolylazo-*o*-toluidine (*o*-aminoazotoluene) in olive oil.

The present report is the first on the induction of fibrosarcomas in rats by subcutaneous injection of *N,N*-dimethyl-*p*-(1-naphthylazo)aniline. Induction of the tumors after only 2 injections of the azo dye is noteworthy. Another notable feature is that these tumors arose only in females, although the transplants grew readily in males. King and Lewis (*6*) injected aromatic hydrocarbons subcutaneously in male and female rats and induced fibrosarcomas in both sexes, although tumors appeared in males at an earlier date than in females. Mulay and Firminger (*7*) induced fibrosarcomas in male Osborne-Mendel rats by feeding *N,N*-dimethyl-*p*-(1-naphthylazo)aniline. It was suggested in their discussion that the pathologic changes produced in the liver by prolonged injection of the carcinogen was a possible factor in the development of the fibro-

sarcomas, since estrogens would accumulate in the male rats because the pathologic liver was unable to dispose of the steroids properly. Gardner (*8*) cited the development of many types of mammary tumors in both male and female rats of certain strains given estrogens in large amounts.

Since spontaneous subcutaneous tumors are rare in old Osborne-Mendel rats and are never seen in young animals, it is concluded that *N,N*-dimethyl-*p*-(1-naphthylazo)aniline was responsible for the induction of these tumors. In the present study the amount of DAN, 25 mg. in 5 cases and 125 mg. in the sixth, was so small as to preclude the possibility of impairment of liver function. The absence of tumors in male rats suggests that estrogens may be necessary for the induction of subcutaneous sarcomas with this carcinogen. The growth of transplants in male rats indicated that the tumors were not estrogen-dependent.

Summary

Among 10 male and 10 female rats that received weekly subcutaneous injections of 0.5 ml. of a 2.5 percent solution of *N,N*-dimethyl-*p*-(1-naphthylazo)aniline in corn oil and were under observation for 20 months, 5 sarcomas and 1 carcinoma developed in the females. The sarcomas were large subcutaneous tumors that arose at the site of injection; they averaged 5 cm. in diameter and 55 gm. in weight. In 4 cases the tumors were multiple (2 separate nodules). The sarcomas were grossly circumscribed but microscopically invasive into adjacent tissues and were composed chiefly of large oval, elongated, or spindle cells. Three of the tumors were successfully carried through several generations. After the second transplant generation, the tumors usually metastasized to the lungs. The carcinoma was small and probably of mammary origin. It was composed of proliferating ducts lined by atypical cuboidal cells from which cords of tumor cells invaded the surrounding stroma.

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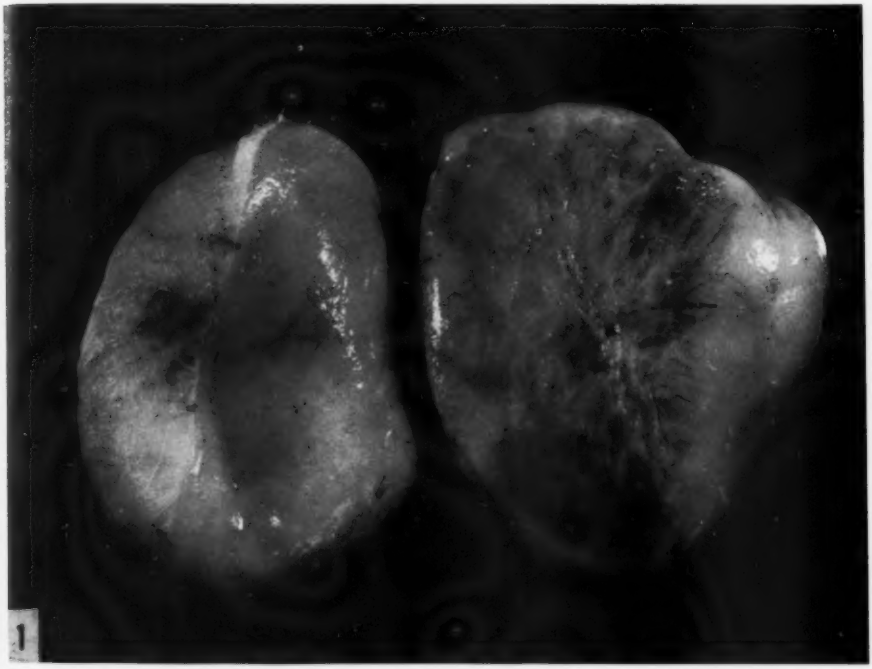
PLATES

PLATE 73

FIGURE 1.—Cut surface of subcutaneous fibrosarcoma showing circumscribed margins and dark areas of liquefaction necrosis. × 1

FIGURE 2.—Central portion of tumor illustrating variation in cell morphology and edematous stroma. × 260



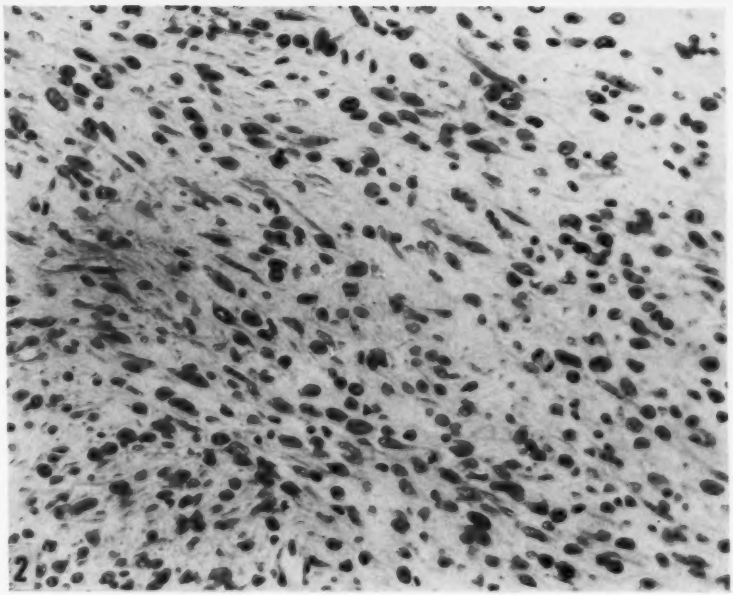


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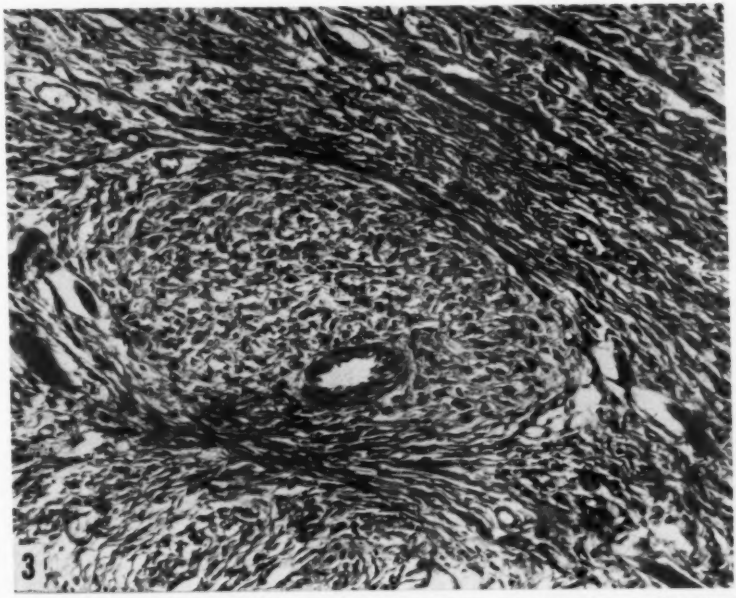
PLATE 74

FIGURE 3.—Sarcoma showing tissue that is more cellular and better preserved about blood vessels. Surrounding striated muscle has been invaded. × 160

FIGURE 4.—Tumor cells forming interwoven bundles. Numerous mitotic figures are present, many of them atypical. × 150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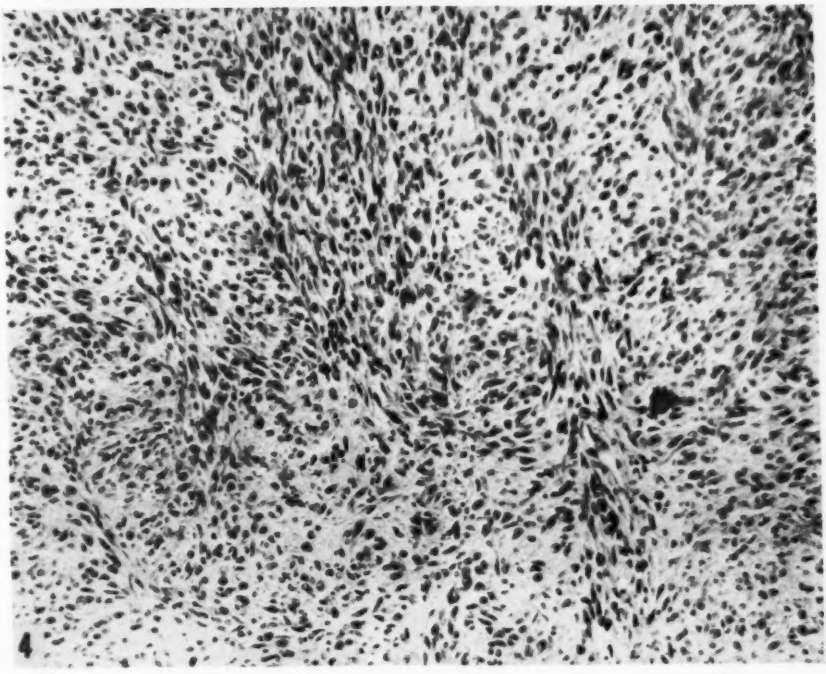
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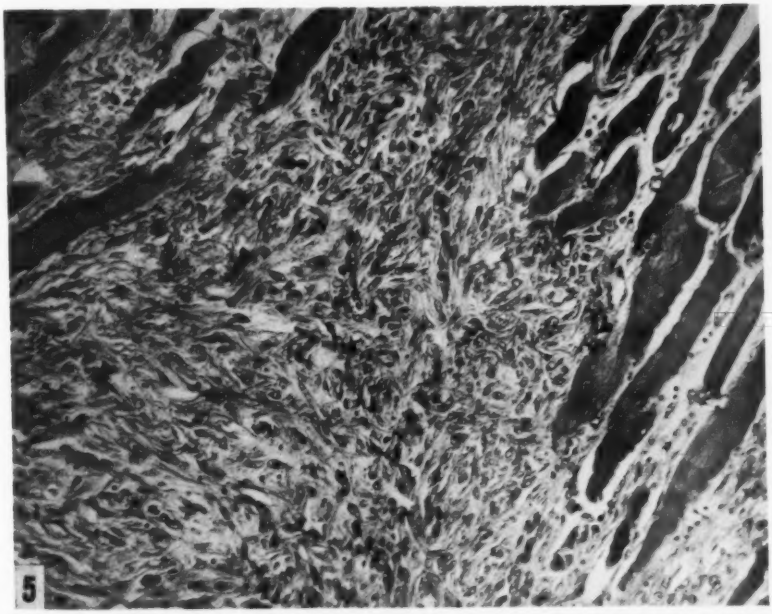
PLATE 75

FIGURE 5.—Invasion of striated muscle by malignant fibroblastic cells. × 230

FIGURE 6.—Fibrosarcoma in the sixth transplant generation. Morphologically the cells are similar to, but more pleomorphic and hyperchromatic than, the original sarcoma. × 290



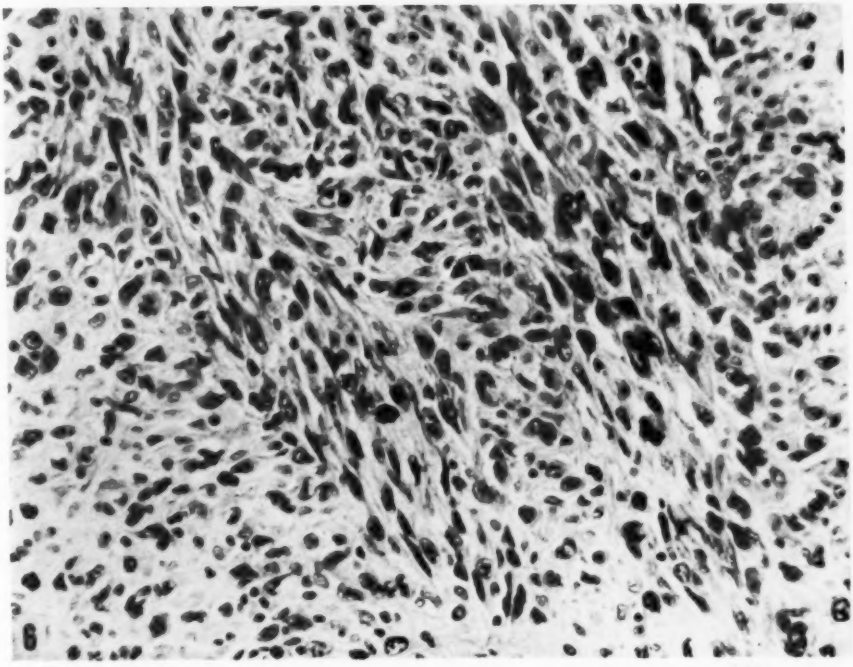
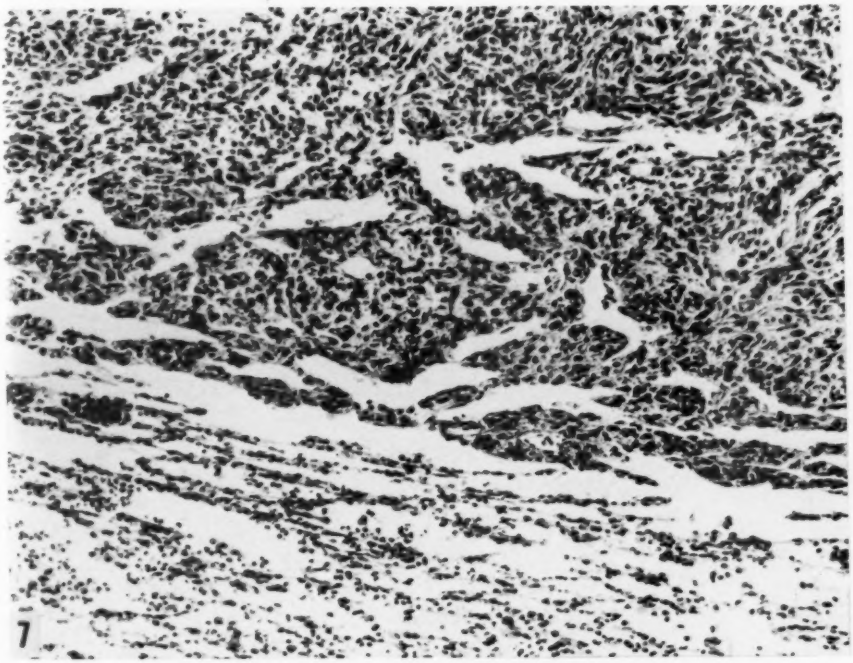


PLATE 76

FIGURE 7.—Fibrosarcoma metastatic to lung in third transplant generation. An area of compressed lung tissue is visible at the lower margin of the photograph. × 150

FIGURE 8.—Carcinoma at the site of injection. Cords of neoplastic epithelial cells invade the fibrous stroma. × 160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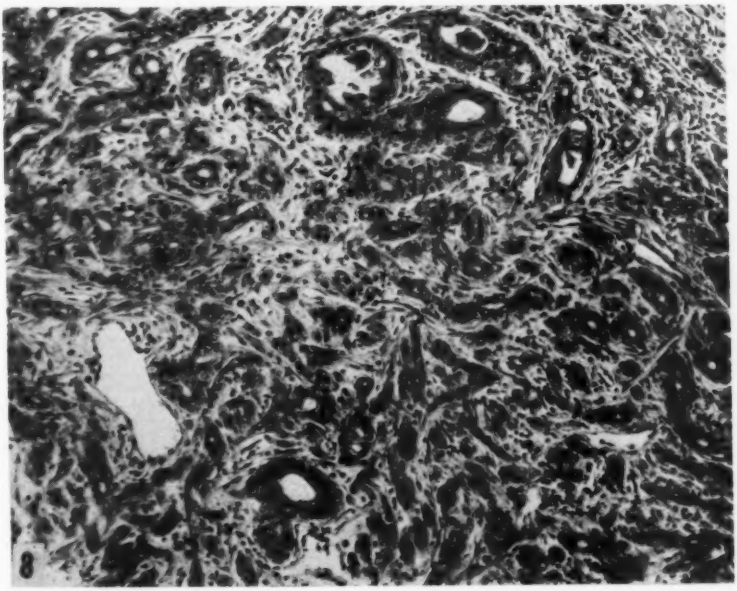
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Squamous Papilloma in the White Croaker[1]

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The pollution of rivers, streams, and coastal sea waters, with industrial effluents containing carcinogenic hydrocarbons, theoretically introduces a potential environmental hazard. Hueper and Ruchhoft (*1*) have reported that the eluates of carbon adsorbates of raw water, polluted with oil-refinery effluent, were weakly carcinogenic for the skin of mice. Cahnmann and Kuratsune (*2*) have demonstrated the presence of several polynuclear aromatic hydrocarbons in oysters taken from waters polluted with ship oil and industrial sewage. Shimkin *et al.* (*3*) succeeded in isolating benzo[*a*]pyrene from barnacles collected from coastal waters. In view of the reported paucity of spontaneous tumors in marine animals from their natural habitat, the occurrence of papillary lesions on the lips of several fish, collected from polluted Pacific waters off the southern California coast, is worthy of comment.

Method

During a 20-day period (May 23–June 20, 1956) 3,857 bony fishes (41 species) and 3,503 cartilagenous fishes (13 species) were taken with trawling gear from Santa Monica Bay, California. Three hundred and fifty-three of them were white croakers or kingfish (*Genyonemus lineatus*). All specimens were examined grossly for the presence of abnormalities. Ten of the fish, all white croakers, were found to have tumor-like lesions about the mouth. These fish were taken from areas designated by the California Department of Fish and Game as P-15 and P-16. Histologic examination of the tumors was undertaken in 3 representative specimens.

Gross Description and Histology

Specimen 1.—A white croaker, 265 mm. in total length, was taken at a depth of 5 fathoms. The upper lip was deformed by 3 firm, round, slightly nodular nonmobile masses. The surfaces of the lesions, in contact with the lower lip, were flattened. The tumors measured

[1] Received for publication March 7, 1957.

[2] Grateful recognition is given to Mr. John Fitch of the California Department of Fish and Game for obtaining the specimens for this report.

10 × 15, 5 × 5, and 8 × 5 mm. Routine sections of the tumors, grossly uninvolved skin, and all visceral organs were taken for histologic studies.

Specimen 2.—This fish measured 240 mm. in total length. Two tumors were present on the upper lip (fig. 1). They measured 20 × 10 and 14 × 9 mm. and were grossly similar in appearance to the lesions described above.

Specimen 3.—A white croaker measuring 223 mm. in total length was taken from a depth of 8 fathoms. Lesions grossly similar to those described were noted on the upper lip at either angle of the mouth. These measured 2 × 3 and 8 × 10 mm.

All lesions were characterized by marked diffuse squamous epithelial hyperplasia (fig. 2). The resulting thickened epithelium was entirely contained within the basement membrane. Normal maturation and polarity of the cells were observed throughout (fig. 3). Isolated nests of epithelial cells were noted in the subepithelial layer. These were arranged in whorls not unlike those seen in epithelial pearls with keratin cores (fig. 4). No invasion or other evidence of malignant neoplastic change was observed. Minimal infiltration of round cells was noted in the subepithelial layers. Inclusion bodies were not demonstrable. Multiple sections of skin and visceral organs revealed no significant lesions.

Discussion

The epidemiologic distribution of papillomas of the lips and epidermis in fishes varies considerably. Papillomas of the epidermis have been described in a number of the lower vertebrates (*4*). Oral papillomas have been reported in the climbing perch (*Anabas scandens*) (*5, 6*), dwarf gourami (*Colisa lalia*) (*5, 6*), European barbel (*Barbus fluviatilis*) (*7*), smelt (*Osmerus eperlanus*) (*8*), eel (*Anguilla vulgaris*) (*9*), halibut (*Hippoglossus hippoglossus*) (*10*), rock bass (*Amblopitis rupestris*), and goldfish (*Carassius auratus*) (*11*). Tumors of this type may occur in response to either mechanical, infectious, or chemical irritation. Nigrelli (*5*) has noted that ulcerative lesions often appear on the snouts of aquarium fishes. These probably arise following repeated collisions with the sides of the tanks. While the majority of these ulcers heal, an occasional tumor develops at the site of or adjacent to the site of the ulceration.

Various papillomas in fishes have been attributed to a virus or virus-like agent (*6–8, 12, 13*).

Specific ecologic considerations require an assessment of water pollution as a factor in the pathogenesis of the papillomas reported. The observation is significant that 10 of the 353 white croakers, taken from waters approximately 2 miles from the outfall of a sewage-treatment plant, bore lesions about the mouth. In contrast, no similar lesions were noted in 1,116 white croakers taken from nonpolluted waters 50 miles away. Lucké and Schlumberger (*14*) noted that nearly all of the affected catfish in their study were from the grossly polluted Schuylkill and Delaware Rivers. These investigators could not exclude the possibility that the lesions were induced by chemical carcinogenic agents in the water. The

abrasions and partial epithelial denudation of the lips of the catfish, which may follow their digging into the river bed, perhaps facilitated carcinogenic activity.[3] The white croaker is similarly a bottom feeder and swimmer. He may dig into the mud and sand and abrade his lips in much the same manner as the catfish. White croakers are found in great numbers in marine waters polluted by domestic and industrial wastes.

The pollution of water with known and suspected carcinogenic agents, incidental to the industrialization of society, was suspected by Hueper in 1942 (*15*). During the intervening years this suspicion has been confirmed. Furthermore, tumors have been demonstrated in marine animals taken from a polluted aquatic environment.

In view of the low incidence of spontaneous tumors in marine fishes taken from their natural habitat, the findings herein reported serve to emphasize the need for additional studies of polluted marine waters. These studies should include a search for the presence of carcinogenic agents and tumor-bearing animals.

Summary

Oral papillomas have been described in fishes.

The gross morphology and histology of squamous papillomas are described in 3 white croakers (*Genyonemus lineatus*).

The role of water pollutants as a possible factor in the etiology of these tumors is discussed.

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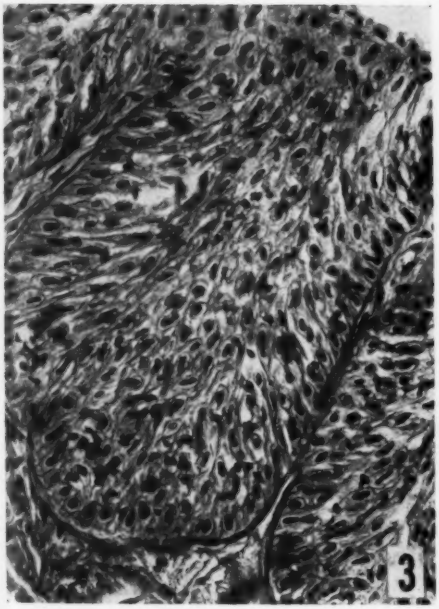
PLATE 77

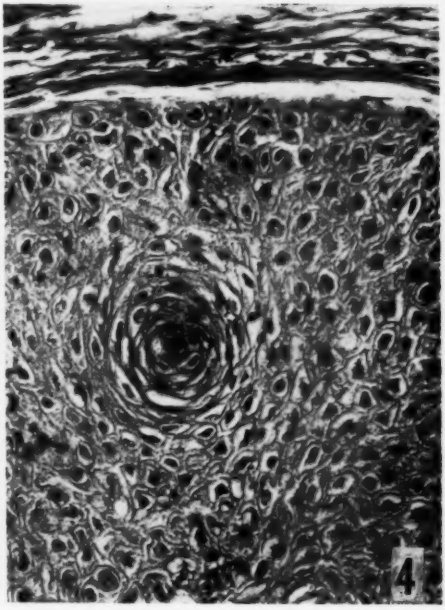
FIGURE 1.—Gross appearance of 2 papillomas on the upper lip of a white croaker.

FIGURE 2.—Section through the larger of the 2 papillomas showing the diffuse, marked, squamous epithelial hyperplasia. Hematoxylin and eosin. × 75

FIGURE 3.—Section showing acanthosis with intact basement membrane. Hematoxylin and eosin. × 225

FIGURE 4.—Subepithelial layer showing epithelial pearl formation. Hematoxylin and eosin. × 225





423744—57——14

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423744—57——15

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INDEX OF AUTHORS

Volume 18

January 1957 • June 1957

Journal of the National Cancer Institute

Page numbers in italics indicate senior authorship

Andervont, Howard B., *1*
Asano, K., 373

Barrett, Morris K., *57*
Beard, Dorothy, *231*, 831
Beard, J. W., 231, 831
Beaudreau, George S., 231, 831
Bittner, John J., *65*
Bonar, Robert A., 231, *831*
Brownell, Leona W., 579
Burstone, M. S., *167*

Canter, Harry Y., 1
Cole, Leonard J., 127
Congdon, Charles C., *603*, 615
Crespi, Henry L., 641

Dunn, Thelma B., *587*

Earle, Wilton R., 701, 709
Eichwald, Ernst J., 473
Eriksen, Nils, 271
Evans, Virginia J., 701, 709

Feldman, Michael, *529*
Ferrigno, M. A., 757
Finkel, Asher J., 641
Fitzgerald, D. B., *83*
Fong, Conrad T. O., *271*
Fugmann, Ruth A., 201
Fujisaku, S., 373
Fukushima, I., 373

Gengozian, N., 603
Ginsberg, Harold S., 487
Greenhouse, Samuel W., 291
Griffin, A. Clark, 693
Groupé, Vincent, *507*

Habermeyer, John G., 127
Hansen, Walter H., 57
Hartwell, J. L., 83
Hasterlik, Robert J., 641
Heston, W. E., *779*
Highsmith, Eleanor M., 285
Hill, J. H., *291*, *301*, *307*, *315*, *323*, *335*
Hitchcock, Claude, R., *795*
Hobbs, Gwendolyn Likely, *701*
Hoch-Ligeti, Cornelia, *661*
Hollcroft, Joanne, *615*
Hsu, T. C., *463*
Hummel, K. P., 719
Hyatt, George W., 709

Jenkins, Robert J., 749

Kahler, H., *217*
Kammer, A. G., 41
Kamoi, M., 373
Katz, Joseph J., *641*
Klein, Eva, 173
Klein, George, 173
Kotin, Paul, 857
Kurihara, M., 373

Law, L. W., 77, 413
Leiter, J., 83
Liebelt, Annabel G., *209*
Lijinsky, William, *687*
Lippincott, Stuart W., 271
Littell, Arthur S., 487

Lloyd, Bolivar J., Jr., 217
Lorenz, Egon, 615

MacLean, Lloyd D., 795
Main, Joan M., 769
Makinodan, T., 603
Malinský, J., 359
Miller, Eliza, 615
Moloney, John B., *615*
Moorhead, Paul S., 463
Morris, Harold P., *101*
Mulay, A. S., *843*

Nagaike, H., 373
Nowell, Peter C., *127*
Noye, Y., 373

O'Gara, R. W., 843
Osgood, E. E., *155*

Peacock, Andrew C., *277*, *285*
Perry, Vernon P., *709*
Poel, William E., *41*
Pontén, Jan, *443*
Potter, Michael, *413*, 587
Powers, O. H., 145
Prehn, Richmond T., *769*
Prince, Alfred M., *487*

Ranadive, Kamal J., 555
Rauscher, Frank J., 507
Rice, Mary E., 117
Richardson, Flavia L., *813*
Ringertz, Nils, *173*
Roan, Patricia L., 127
Russell, Findlay E., *857*

Sachs, Leo, 529
Saffiotti, Umberto, 687
Saito, S., 373
Sanford, Katherine K., 701, 709
Šantavý, F., 359
Schneider, Walter C., *569*, *579*
Schweisthal, Robert, 615
Segi, M., *373*
Seidlová-Mašínová, V., *359*
Shack, Joseph, *749*
Shacter, Bernard, *77*, *261*, *455*
Shapiro, Daniel M., *201*
Sharp, D. G., 231, 831
Shelton, Emma, *117*
Shimkin, Michael B., 1
Shubik, Philippe, 687
Smith, Robert Lincoln, *385*, *397*
Spain, James D., *693*
Spjut, Harlan J., *473*
Steffee, C. H., 779
Stevens, L. C., *719*
Sullivan, W. Albert, 795

Thomson, John F., 641
Thorell, Bo, 443
Trentin, J. J., *757*

Uphoff, Delta, 615

Van Lancker, J., *407*
Velat, Clarence A., 101

Wagner, Billie P., 101
Waravdekar, Susheela S., *555*
Waravdekar, V. S., *145*
Williams, George Z., 277

Zilber, L. A., *341*

INDEX OF SUBJECTS

Volume 18

January 1957 • June 1957

Journal of the National Cancer Institute

Achlorhydria and gastric atrophy, relationship to development of gastric cancer, 795
Adenocarcinoma 755, Bagg-Jacksen, drug synergism in treatment of mice bearing, 201
Aminobiphenyls, induction of tumors in rats with, 101
Announcements:
  Fourth International Gerontological Congress, 154
  Responsibilities of the Medical Profession in the Use of X Rays and other Ionizing Radiation, 481
  Seventh International Cancer Congress, 153
  Symposium on Fundamental Cancer Research, 154
Antigen(s), tumor-specific, studies on in guinea pigs, 341
Antimetabolite(s), development of resistance of plasma-cell tumors in mice to, 413
Ascites tumor(s): *see also* Ehrlich ascites, Krebs-2, Leukemia, Mast-cell, Sarcoma
  conversion in mice of MC1M sarcoma from solid form to, 173
Avian erythroblastosis: *see* Virus
Azaguanine-deaminase activity of leukemia sublines in mice, 77
Azaserine, development of resistance of plasma-cell tumors in mice to, 413

Benz[*a*]anthracene (1,2-benzanthracene), identification as constituent of and possible relationship to carcinogenicity of creosote oil, 687
Bile, changes in constituents of, compared with liver morphology during azo-dye carcinogenesis in rats, 693
Bone marrow, effect of on reticular tissues of X-irradiated mice, 603
Breast tumors: *see* Mammary tumor

Carcinogen(s): *see* Aminobiphenyls, Benz[*a*]anthracene, Contraceptives, Creosote oils, Dimethylaminoazobenzene, *N,N*-Dimethyl-*p*-(1-naphthylazo)aniline, *N*-2-Fluorenylacetaminde, Hydrocarbons, 3-Methylcholanthrene
Carcinogenesis:
  carcinogenic activity of isosteres of known carcinogens tested in mice, 555
  epidermal, by creosote oils, 41, 687
  mast-cell neoplasm in mice induced with methylcholanthrene, 577
  tumor production with spermicidal contraceptives in rats on full and low-protein diets, 661
  unifying concept of etiology of cancers, 155
Carcinoma(s): *see* names and types of
Cervix, cancer of, possible role of spermicidal contraceptives in production of, 661
Chemotherapy, combination, drug synergism in tumor-bearing mice given, 201
Chromosome studies:
  heteroploidy in human cells *in vitro*, 463
Cigarette and cigar smoke, studies on with electron microscope, 217
Clone(s):
  establishment of from a single mouse liver cell of a strain adapted to grow in chicken plasma clot, 701
  of human cells, established from normal human skin, 709
Contraceptives (spermicidal), tumor production in rats on full and low-protein diets with, 661

Creosote oil(s):
chemical constitution of and carcinogenic activity in mice of, 687
epidermal carcinogenesis induced with, 41
Cysteine and anoxia, protection against total-body X irradiation in mice with, 615

Deoxypentose nucleic acid, electrophoretic properties of, from lymphoma #1 and calf thymus, 749
Deoxyribosidic compounds:
concentrations of in regenerating rat liver, 579
isolation and identification of, in Novikoff hepatoma, 569
Deuterium, biological effects of on mice, with a section on neoplastic processes, 641
Diacetylaminofluorene: *see* *N*-2-Fluorenylactamide
Diagnostic tests: *see also* Techniques
for cancer, evaluation of, 269–339
for gastric cancer based on achlorhydria, 795
6-Diazo-5-oxo-L-norleucine (DON), development of resistance of plasma-cell tumors in mice to, 413
Dimethylaminoazobenzene:
3′methyl-4———, comparison of changes in bile constituents and liver morphology in rats during carcinogenesis with, 693
N,*N*-Dimethyl-*p*-(1-naphthylazo)aniline (*p*-dimethylaminobenzene-1-azonaphthalene), induction of sarcomas in rats with, 843

Ehrlich ascites tumor(s):
biossay of cells of, 487
influence of sulfhydryl reagents *in vitro*, on cells of, 455
Enzyme(s):
azaguanine-deaminase activity of several leukemia sublines in mice, 77
choline-oxidase activity of mouse leukemic cells, 261
diphosphopyridine nucleotide, synthesis of, by normal and tumor tissues of mice, 145
esterase, cytochemical localization of, 167
measurement of succinoxidase activity by tetrazolium reduction in mouse tissues, 117
Erythroblastosis: *see* Virus
Estrogen(s):
effect on testicular tumors of treatment with, 1
incidence of mammary and pituitary tumors in hybrid mice treated with stilbestrol, 831
Ethionine, synergism with a possible folic acid antagonist in chemotherapy of mice, 201

Fibrosarcomas, methylcholanthrene-induced, production of isologous immunity in inbred mice to, 769
Filipinos: *see* Statistics
Fish, possible role of water pollutants in etiology of squamous papilloma of, 857
N-2-Fluorenylacetamide, induction of mammary tumors in rats with, 473
Folic acid, synergism with a possible antagonist of, in chemotherapy of mice, 201

Gastric cancer, relationship of achlorhydria and gastric atrophy to development of, 795
Genetics:
effect of transplantation on adult and fetal lung tissue from mice genetically susceptible and resistant to lung tumors, 779
factors influencing successful skin grafting in inbred mice, 209
Grafts, skin, in inbred mice, genetic factors influencing, 209

Hawaiians: *see* Statistics
Hepatoma (Novikoff), isolation and identification of some deoxyribosidic compounds in, 569
Hormones: *see* Estrogen(s)
Hydrocarbons, carcinogenic, biological testing of sulfur isosteres of, 555

Indians of U. S.: *see* Statistics
Infectious ectromelia: *see* Mouse pox
Immunology:
antibody response in mice to homografts of 3 sarcomas, 529
antigens, tumor-specific, studies on in guinea pigs, 341
production of isologous immunity to methylcholanthrene-induced sarcomas in inbred mice, 769

Japanese: *see* Statistics

Krebs-2 carcinoma, effect of deuterium on growth rate in mice of, 641

Leukemia:
chicken, histogenesis of virus-induced, 443
lymphoid tumor(s):
lymphocytic leukemia
azaguanine-deaminase activity of in mice, 77
——— P1534, effect of deuterium on growth rate of in mice, 641
lymphoma #1, electrophoretic properties of DNA from, 749
lymphosarcoma 6C3HED, antibody response to homografts of in mice, 529
mouse (L1210)
choline-oxidase activity of cells of, 261
synthesis of diphosphopyridine nucleotide by, compared with normal tissue, 145
Lipides, inhibition of Rous sarcoma virus by oxidation products of, 515
Liver: *see also* Hepatoma
morphology compared with changes in bile constituents during azo-dye carcinogenesis in rats, 693
regenerating, concentrations of deoxyribosidic compounds in, 579
Lung tumors, effect of transplantation on adult and fetal lung tissue from mice genetically susceptible and resistant to, 779

Mammary tumor(s):
agent, activity of in mice of different ages and their offspring, 65
incidence of, in hybrid mice treated with stilbestrol, 813
induction of in rats with *N*-2-fluorenylacetamide, 473
Mast-cell tumor in ascitic form, morphology and behavior in mice, 587
3-Methylcholanthrene, production of isologous immunity in inbred mice to sarcomas induced with, 769
N-Methylformamide, development of resistance of plasma-cell tumors in mice to, 413
Mitosis in tumor cells, effect of podophyllin compounds on, 359
Morbidity and mortality: *see* Statistics
Mouse pox, control of by immunization with vaccinia virus, 757

Papilloma, squamous, in fish, possible role of water pollutants in etiology of, 857
Pituitary tumors, incidence of in hybrid mice treated with stilbestrol, 813
Plasma-cell tumors: *see* Reticular tumors
Podophyllin compounds:
dependence of biological effects in rats upon chemical structure of, 359
podophyllotoxin, and deoxypodophyllotoxin, isolation of from leaves of conifers, and tumor-damaging activity, 83

Radiation: *see* X irradiation
Reticular tissue in X-irradiated mice, effect of bone marrow on, 603
Reticular tumors (plasma-cell), in mice, development of resistance to antimetabolites by, 413
Rous sarcoma I: *see* Sarcoma, Virus

Sarcoma: *see also* Fibrosarcoma, Leukemia
——— 37
necrotizing activity of deoxypodophyllotoxin and podophyllotoxin on, 83
synthesis of diphosphopyridine nucleotide by, compared with normal tissue, 145
induction of, in rats with *N,N*-dimethyl-*p*-(1-naphthylazo)aniline, 843
MC1A and MC1M, antibody response of mice to homografts of, 529
MCIM, conversion from solid to ascites form, in mice, 173
Rous ——— I, inhibition of activity of tumor-producing agent by oxidized tumor lipides, 515
Spleen, protection against total-body X irradiation in mice by actual and sham shielding of, 615
Statistics:
morbidity
cancer in Japan, study of in defined geographic area, 373
mortality
recorded and expected, with data on cancer, among
Indians of U. S., 385
Filipinos and Hawaiians, 397
quantitative bioassays of Ehrlich ascites-tumor cells, 489
Sulfhydryl reagents, influence of on Ehrlich ascites-tumor cells, 455

Techniques:
cytochemical localization of esterase, 167
diagnostic tests for cancer in man, evaluation of
chromate-serum precipitation reaction, 315
fibrin proteolysis by serum (Fuchs test), 285
fluorescence of urine in cancer diagnosis, 335
glycolysis of hemolysates, effect of carotene on, 323
hemolysis of sheep erythrocytes by human serum (Zuccala test), 301
infrared spectroscopy of crystalline albumin in serums, 271
serofloccculation reaction (Penn-Hall test), 277
serum lactic dehydrogenase, cancer selectivity of, 307
turbidimetry, determination of plasma-protein pattern by, 291
electron microscopy, studies on tobacco smoke with, 217
measurement of succinoxidase activity by tetrazolium reduction in mouse tissues, 117
Teratoma testis, spontaneous congenital, description of in mice, 719
Testicular tumors, effect of estrogenic stimulation on, 1
Therapy: *see* Chemotherapy
Thymus, calf, electrophoretic properties of DNA from, 749
Tissue culture:
clones of cells established from normal human skin, 709
growth of mouse liver cells on chicken plasma clot and establishment of clone from single cell, 701
Transplantation: *see also* Grafts
effect of, on adult and fetal lung tissue from mice genetically susceptible and resistant to lung tumors, 779
of homografts of 3 sarcomas, antibody response in mice to, 529
Tumor(s): *see also* Adenocarcinoma, Ascites, Ehrlich ascites, Fibrosarcoma, Hepatoma, Krebs-2, Leukemia, Lung Mammary, Mast-cell, Pituitary, Papilloma, Recticular, Sarcoma, Teratoma, Testicular
induction of, with aminobiphenyls in rats, 101
immunity in mice, undulations in time-response curve, 57
Tumor-damaging agents: *see* Podophyllin compounds

Virus:
avian erythroblastosis
antigenic constitution and relation to myeloblastosis virus 1, 231
*p*H and thermal stability of, 831
determination of viral and tissue antigens in tumors in guinea pigs, 341
histogenesis of chicken leukemia induced by, 443
Rous sarcoma
inhibition of tumor-producing activity of, by oxidized tumor lipides, 515
growth curve of, and relationship of infecting dose to yield of virus in chick brain, 507
vaccinia, control of mouse pox by immunization with, 757

X irradiation:
correlation of survival and regeneration of hematopoietic tissue in rats given lethal doses of, 407
effect of deuterium in mice on sensitivity to, 641
effect of rat bone-marrow injection on reticular tissues of mice exposed to, 603
total-body, survival of mice with and without protection following, 615

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CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL. 18 • NO. 6 • JUNE 1957

Page

Immunity to methylcholanthrene-induced sarcomas. *Richmond T. Prehn* and *Joan M. Main* 769

Development of tumors in fetal and adult lung transplants. *W. E. Heston* and *C. H. Steffee* 779

The secretory and clinical aspects of achlorhydria and gastric atrophy as precursors of gastric cancer. *Claude R. Hitchcock, Lloyd D. MacLean,* and *W. Albert Sullivan* 795

Incidence of mammary and pituitary tumors in hybrid mice treated with stilbestrol for varying periods. *Flavia L. Richardson* 813

Virus of avian erythroblastosis. IV. *p*H and thermal stability. *Robert A. Bonar, Dorothy Beard, George S. Beaudreau, D. G. Sharp,* and *J. W. Beard* .. 831

Transplantable sarcomas induced in rats by *N,N*-dimethyl-*p*-(1-naphthylazo)-aniline. *A. S. Mulay* and *R. W. O'Gara* 843

Squamous papilloma in the white croaker. *Findlay E. Russell* and *Paul Kotin* .. 857

Acknowledgment 863

Index of authors 865

Index of subjects 867

5786